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December 1962 through February 1963

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BIO-ORGANIC CHEMISTRY QUARTERLY REPORT

December 1962 through February 1963

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March 29, 1963

1. A VERSATILE SOLVENT TO REPLACE PHENOL FOR THE PAPER CHROMATOGRAPHY OF RADIOACTIVE INTERMEDIARY METABOLITES

Gerald J. Crowley, V. Moses, and Johannes Ullrich

Analysis of the products of radioactive-tracer feeding experiments in biological systems has depended to a considerable extent on paper chromatography. Of the many solvent systems proposed, the one devised by Benson et al.,[1] based on the use of phenol-water followed by n-butanol—propionic acid—water, has many advantages for the separation of such biological intermediates as sugars, sugar phosphates, amino acids, and carboxylic acids. Nevertheless, phenol-water mixtures have several inherent drawbacks; Mizell and Simpson[2] recently surveyed some of these, and to this list we might add both the corrosive action of phenol on human skin and the fact that many amino acids show signs of decomposition when chromatographed in phenol-containing solvents.[3,4]

The quest for a solvent to replace phenol led us to consider some of those mentioned in the literature,[2,5,6] but none was suitable for our purpose. Eventually interest centered on solvents based on isobutyric acid, ammonia, water, and ethylenediaminetetraacetic acid (EDTA).[7] Although this solvent was not entirely satisfactory as originally formulated[7]--because such slow-running hydrophilic substances as sugar phosphates migrated too far and left empty much of the chromatogram near the origin--the mixture seemed promising as a basis for further experimentation. There were two approaches

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1. A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, J. Am. Chem. Soc. 72, 1710 (1950).
2. M. Mizell and S. B. Simpson, J. Chromatog. 5, 157 (1961).
3. A. K. Huggins and V. Moses, Nature 191, 668 (1961).
4. V. Moses, J. Chromatog. 9, 241 (1962).
5. R. E. Kay, D. C. Harris, and C. Entenman, Arch. Biochem. Biophys. 63, 14 (1956).
6. V. M. Brenner and A. Niederwieser, Experientia 16, 378 (1960).
7. E. Tyszkiewicz, Anal. Biochem. 3, 164 (1962).

towards improving both resolution and the spread of compounds along the whole length of the chromatogram: manipulation of the pH, and an enhancement of the nonaqueous character of the liquid. The pH of the original isobutyric acid–ammonia–water–EDTA solvent is about 4.2; replacement of some of the water with sufficient ammonia solution to raise the pH to 6.8 resulted in a relatively viscous and very slow-running solvent, in which the resolution and spread actually deteriorated.

The first attempts to reduce the aqueous content of the solvent consisted of replacing the ammonia solution, partially or completely, with such organic bases as pyridine and trimethylamine in various combinations, always maintaining the pH at 4.2. A further variation was to saturate the original solvent mixture with toluene. It soon became apparent that the use of these substances did little to improve the chromatographic separation; however, we were stimulated to invent quite a complex vocabulary to describe the olfactory impact of the products. Subsequent alteration of the solvent mixtures was directed to a partial replacement of the water by one of a number of simple alcohols: ethanol, n-propanol, isopropanol, and n-butanol. This led to marked improvements in solvent characteristics, though no single alcohol was completely satisfactory in this regard. Mixing of those alcohols which looked promising when used singly was then attempted. After numerous trials, a mixture (hereafter designated "semistench") was prepared which possessed the most suitable solvent properties for our purposes:

EDTA	1.2 g
17 N ammonia soln.	100 ml
Water	950 ml
n-propanol	350 ml
isopropanol	75 ml
n-butanol	75 ml
isobutyric acid	2,500 ml

Standing at room temperature for 24 hr appeared to establish esterification equilibria between the alcohols and isobutyric acid. The ammonium isobutyrate buffered the mixture at a pH of about 4.0. Aging of the mixture, even for a period of weeks, effected no discernible change in resolution properties. On the other hand, comparison of mixtures aged for at least 24 hr with corresponding solutions freshly prepared showed that aging for one day is mandatory for optimal results. No attempt was made to accelerate esterification either by mild heating or by refluxing, since it was quite convenient to prepare the solvent at least a day before use.

For chromatographic development, the two-dimensional descending technique was employed. The semistench solvent was used for the first dimension; the second dimension was developed with n-butanol–propionic acid–water.[1] Standard Whatman No. 4 chromatography paper was used exclusively; neither oxalic acid-washed paper nor paper rinsed in an alkaline EDTA solution (pH 8.5) was more effective. Both solvents were allowed to run to the edges of the paper; this took (at 23°) 10 to 14 hr for the first dimension (about 50 cm) and 7 to 10 hr for the second (about 38 cm), depending mainly on the batch of paper. Known compounds were localized by spraying or dipping the

paper in the appropriate reagents. Radioactive substances were found by radioautography, positive identification then being made by cochromatography with authentic marker compounds.

In our actual experimental conditions, the compounds of interest are chromatographed in the presence of cell extracts. Since there is probably interaction between compounds in mixtures, the chromatographic parameters of all the compounds investigated were measured by mixing each authentic substance with an ethanol-water extract of Chlorella pyrenoidosa cells or of spinach chloroplasts which had been allowed to fix $^{14}CO_2$ photosynthetically.[1] Table 1-I lists the R_ϕ values for all the compounds studied with respect to aspartic acid.

Table 1-I. $R_{\text{aspartic acid}}$ values in semistench (A) and in n-butanol–propionic acid–water (B)

Amino acids and peptides	A	B
α-Alanine	2.49	1.30
β-Alanine	2.65	1.66
γ-Aminobutyric acid	3.46	2.04
Arginine	2.90	1.14
Asparagine	1.50	1.00
Aspartic acid	1.00	1.00
Citrulline	1.85	1.13
Cysteine	1.01	0.63
Cystine	0.81	0.63
Glutamic acid	1.25	1.08
Flutamine	1.58	0.92
Glutathione, oxidized	0.10	0.55
Glutathione, reduced	1.07	0.46
Glycine	1.82	0.96
Histidine	3.66	0.42
Leucine	3.92	2.30
Isoleucine	3.50	2.77
Lysine	2.82	0.67
Methionine	3.23	2.33
Phenylalanine	3.62	2.55
Proline	3.07	1.65
Serine	1.70	0.94
Threonine	2.22	1 20
Tryptophane	2.68	1.77
Tyrosine	2.36	1.64
Valine	3.44	2.61
Carboxylic acids, hydroxy- and oxo-carboxylic acids		
cis-Aconitic acid	0.89	1.48
cis-Aconitic anhydride	0.87	1.76
Citric acid	0.79	1.46
Isocitric acid	0.84	1.57
Fumaric acid	1.13	1.83

Table 1-I (Cont.)

Glyceric acid	1.43	1.52
Glycolic acid	1.77	1.73
Glyoxylic acid	1.83	1.55
Lactic acid	2.47	2.16
Malic acid	1.09	1.58
α-Oxoglutaric acid	1.12	1.51
Pyruvic acid	1.75	1.60
Succinic acid	2.00	1.87
Tartaric acid	0.53	0.94
Sugars		
Arabinose	1.53	1.26
2-Desoxyribose	2.65	1.96
Dihydroxyacetone	2.00	2.11
Erythrose	1.71	1.32
Erythrulose	2.19	1.67
Fructose	1.52	1.15
Galactose	1.18	1.02
Glucose	1.20	1.05
Gulose	1.30	1.07
Lactose	0.63	0.64
Lyxose	1.59	1.29
Maltose	0.74	0.74
Mannose	1.17	1.01
Ribose	1.83	1.36
Ribulose	2.13	1.43
Sedoheptulose	1.21	1.07
Sorbose	1.38	1.13
Sucrose	1.26	0.99
Threose	2.28	1.67
Xylose	1.60	1.34
Xylulose	1.95	1.29
Sugar alcohols		
Arabitol	1.75	1.19
Dulcitol	1.47	1.06
Glycerol	3.03	1.91
Mannitol	1.55	1.14
Ribitol	1.53	1.20
Sorbitol	1.53	1.12
Xylitol	1.72	1.15
Uronic acids		
Glucuronic acid	0.72	0.77
Flucuronolactone	1.67	1.37
Aldonic acids		
Ascorbic acid	1.42	1.69
Gluconic acid	0.78	1.04

Table 1-I (Cont.)

Nucleosides		
Adenosine	3.51	1.98
Cytidine	2.54	1.66
Guanosine	1.50	1.37
Inosine	1.42	1.24
Thymidine	2.81	2.47
Uridine	1.67	1.38
Nucleotides		
Adenosine monophosphate	1.35	0.86
Adenosine diphosphate	0.68	0.35
Adenosine triphosphate	0.37	0.23
Guanosine monophosphate	1.35	0.86
Guanosine diphosphate	0.21	0.39
Guanosine triphosphate	0.08	0.22
Inosine monophosphate	0.59	0.49
Thymidine monophosphate	1.27	1.06
Uridine monophosphate	0.91	0.79
Uridine triphosphate	0.10	0.21
Sugar monophosphates		
Dihydroxyacetone phosphate	0.81	0.77
Fructose-6-phosphate	0.55	0.65
Glucose-6-phosphate	0.42	0.57
Hamamelose-6-phosphate	0.51	0.73
Maltose monophosphate	0.35	0.47
Ribose-5-phosphate	0.68	0.75
Sedoheptulose-7-phosphate	0.42	0.57
Sugar diphosphates		
Fructose diphosphate	0.14	0.24
Hamamelose diphosphate	0.14	0.35
Ribulose diphosphate	0.22	0.39
Miscellaneous phosphates		
Phosphoenolpyruvic acid	0.71	1.16
6-Phosphogluconic acid	0.23	0.71
2-Phosphoglyceric acid	0.60	0.90
3-Phosphoglyceric acid	0.53	0.88
Uridine diphosphoglucose	0.44	0.31

The mobilities reported in Table 1-I must be used only as an approximate guide to chromatographic position when compounds are being studied in a complex mixture. These values were obtained by adding two or three known substances at a time to the ^{14}C-labeled plant extract, and relating their position to aspartic acid. Therefore we cannot be certain that compounds having nearly identical mobilities will have the precise relationships to each other indicated in Table 1-I, since we have not simultaneously chromatographed all the listed compounds on one sheet of paper. Slight variations between different chromatograms in a series preclude absolute certainty of chromatographic mobility for any particular substance. The mobilities in two dimensions of more than twenty compounds were measured with respect to aspartic acid on eight replicate chromatograms similar to the one shown in Fig. 1-1. The standard deviation of these measurements showed an average for all the spots of ±2.8% of the means in the semistench solvent, and ±3.9% of the means in n-butanol–propionic acid–water. Thus the values reported in Table 1-I should be regarded as possessing an error of ±6 to 8%.

A radioautogram of a typical chromatogram of ^{14}C-labeled chloroplast extract is shown in Fig. 1-1, compared with a parallel chromatogram of a similar extract run in phenol-water[1] as the first solvent (Fig. 1-2). It will be seen that with the semistench solvent the total area available on the chromatogram is more effectively used than with phenol-water. Both systems have disadvantages resulting from the overlapping of certain compounds which it would be most desirable to have separated. In semistench plus butanol–propionic acid this affects mainly glutamine, glycine, and serine, while in phenol plus butanol–propionic acid, glutamine runs well clear of the other two amino acids, though the latter cannot be separated from glucose. The new system, moreover, results in spots more compact than does phenol, and shows much less variability as far as diffuseness and streaking of the spots is concerned.

In comparing these two solvent systems one effect has consistently been observed, but has not been completely resolved. Using the same cell extract, more spots are separated in the semistench than in the phenol system. We think that these are neither artifacts nor degradation products, but reflect the greater resolving power of semistench. A preliminary study of the action of semistench as far as the decomposition of amino acids is concerned has shown it to be much milder than phenol: the latter causes considerable breakdown of some amino acids.[3,4] The reason for this destruction by phenol is possibly a result of atmospheric oxidation of phenol to form the deep red phenoquinone, a relatively strong oxidizing agent. None of the preservatives generally recommended for phenol chromatography completely inhibits the process, though the addition of α-tocopherol or potassium cyanide to the phenol-water mixture does slow down oxidation.[8]

It must be pointed out that some concern may justly be accorded those substances (acid anhydrides, enolic esters, and other high-energy compounds) which are often more susceptible to ammonolysis than to hydrolysis. It is possible that some of the newly differentiated spots may be products of ammonolysis of certain of the groups mentioned. Although we have neither proven nor disproven this, it should be noted that the versatility of the new solvent

8. Gerald J. Crowley, Dissertation: Invertebrate Serological Responses, University of San Francisco, 1962.

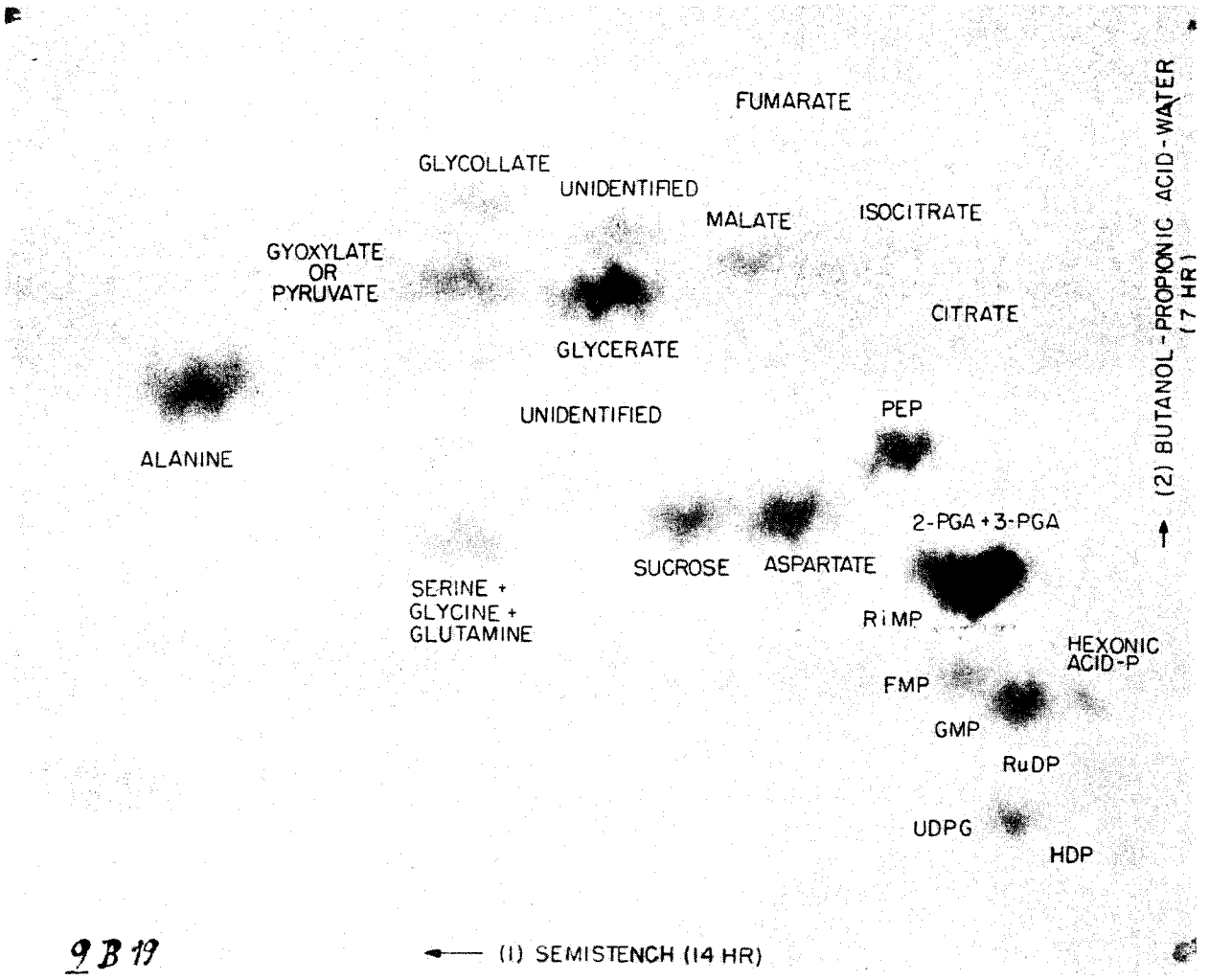

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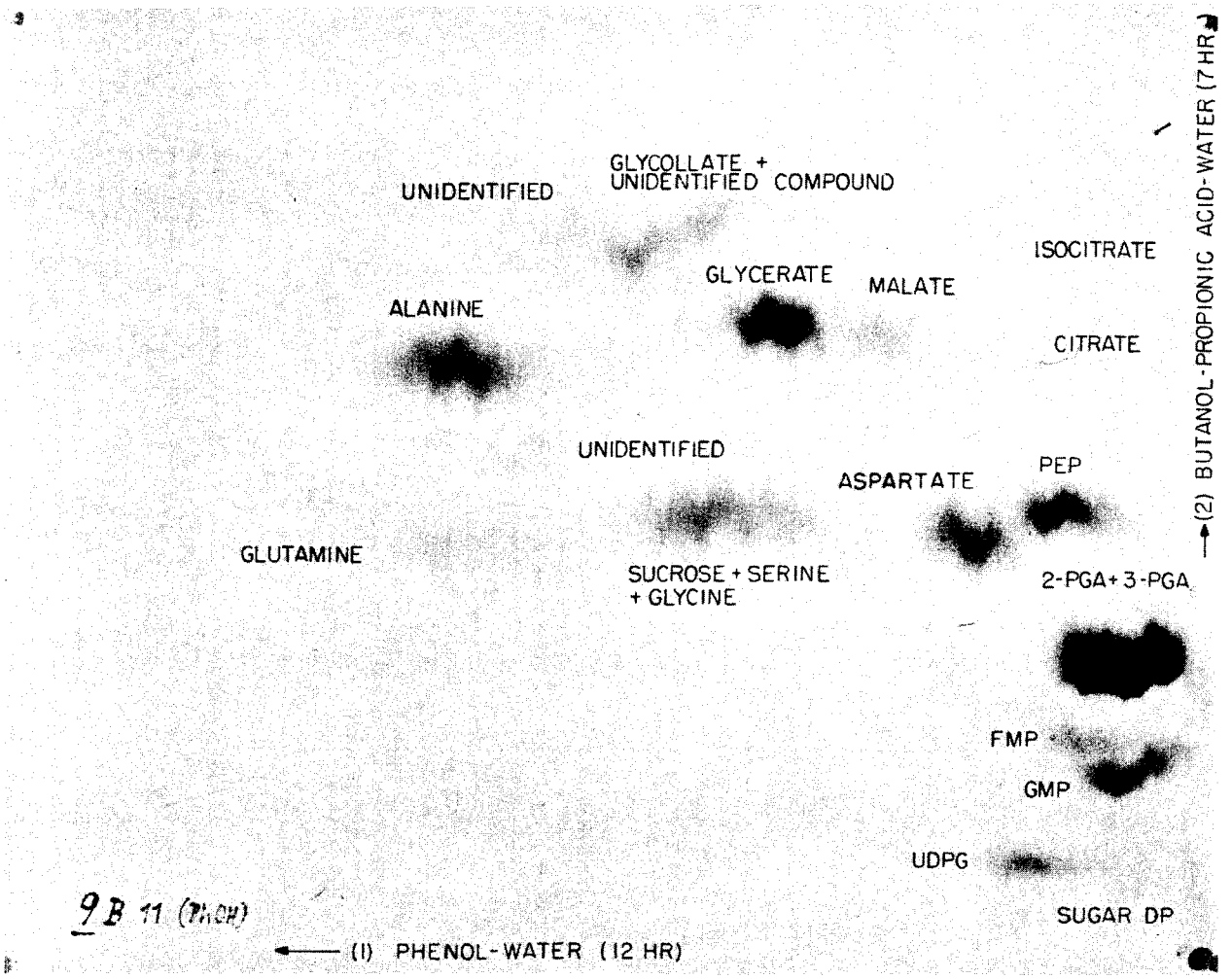
Fig. 1-1. Radioautograph of chromatogram of extract of ^{14}C-labeled chloroplasts, developed to the edges of the paper only with (1) semistench (14 hr) and (2) n-butanol—propionic acid—water (7 hr). Abbreviations: FMP, fructose monophosphate; GMP, glucose monophosphate; HDP, glucose and fructose diphosphates; hexonic acid-P, monophosphate of an unidentified hexonic acid; PEP, phosphoenolpyruvic acid; 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; RiMP, ribose monophosphate; RuDP, ribulose diphosphate; UDPG, uridinediphosphoglucose.



ZN-3767

Fig. 1-2. Radioautograph of chromatogram of same extract as in Fig. 1-1, developed to the edges of the paper only with (1) phenol-water (12 hr) and (2) n-butanol–propionic acid–water (7 hr). Abbreviations: Sugar $\overline{DP}$, sugar diphosphates; others as for Fig. 1-1.

system is such that an equimolar quantity of trimethylamine may be substituted for the ammonia without affecting the chromatographic pattern.[9]

As with the phenol system, chromatograms developed only to the edges of the paper with semistench and butanol–propionic acid show the phosphate esters congregated close to the origin and inadequately separated. Better resolution of these substances may be obtained by overrunning the chromatograms for 40 to 60 hr in semistench and 20 to 24 hr in butanol–propionic acid. However, in this case, the mobilities of the various phosphates with respect to aspartic acid are not the same as when the chromatograms are developed to the edges of the paper only. Table 1-II lists the R_ϕ values under overrunning conditions with reference to 3-phosphoglyceric acid, and a radioautogram is shown in Fig. 1-3. These different mobilities after prolonged chromatographic development may probably be ascribed to three factors: (a) greater esterification in the solvent with increasing time after mixing (this would be of greater significance with butanol–propionic acid–water, as this solvent is freshly prepared and esterification would thus be progressive), (b) differential evaporation of component substances in the mixture; (c) paper-chromatographic separation of the solvent constituents themselves, resulting in a series of bands of varying composition along the chromatogram.

Differences in solvent composition at varying stages after preparation and during one-dimensional chromatograms are shown in Table 1-III. After development the wet chromatogram was cut into strips, 7.5 cm wide, perpendicular to the direction of solvent travel. Liquid was obtained from each strip, as well as from residual solvent in the trough. All these samples were analyzed by vapor-phase chromatography, using a polymetaphenyl ether column at 90° flushed with helium. Quantitative determinations of the separated components were made by measurements of the peak heights. Small amounts of esters would probably have been obscured by the carboxylic acid peak. It will be seen from Table 1-III that n-butanol–propionic acid–water, but not semistench, loses water simply by standing in the trough. Both solvents show changes as they flow down the paper, becoming relatively poor in water with increasing distance traveled. Semistench also becomes relatively poorer in n-butanol and in the acid component.

Some substances, notably carboxylic acids, have different mobilities in butanol–propionic acid, depending on whether the solvent in the first dimension was semistench or phenol-water. This might be explained as a result of the formation of the ammonium salts after development in semistench, the ammonium salts then having lower mobilities than the free acids in butanol–propionic acid. This would be particularly significant for the dicarboxylic and oxocarboxylic acids, all of which are considerably stronger acids than propionic.

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9. Johannes Ullrich, in Bio-Organic Chemistry Quarterly Report, UCRL-9900, Sept. 1961, p. 30.

Two further properties of semistench are worthy of mention. Firstly, the flexibility of the system enables its hydrophilicity to be increased or decreased at will to suit particular circumstances. Increasing the water content at the expense of the alcohols allows the more water-soluble components to migrate further, and vice versa. The second property is of value in preparing substances for use in microbiological assay and in enzymology. No matter for how long a paper chromatogram is dried it is almost impossible to remove all traces of many solvents, even after development with another solvent. Trace amounts of phenol can poison both enzymic and microbiological systems, but traces of isobutyric acid presents no apparent difficulty in this respect.

Table 1-II. Effect of overrunning on R_3-phosphoglyceric acid of phosphates. Chromatograms developed either to edge of paper (12 hr for semistench; 8 hr for n-butanol—propionic acid—water) or overrun for 60 hr in semistench and 20 hr in n-butanol—propionic acid—water (BuPr)

	R_3-phosphoglyceric acid			
	Chromatogram developed to edge of paper only		Chromatogram overrun	
	Semistench	BuPr	Semistench	BuPr
Fructose-6-phosphate	1.03	0.75	0.91	0.73
Fructose diphosphate	0.27	0.29	0.23	0.23
Glucose-6-phosphate	0.77	0.68	0.63	0.66
Phosphoenolpyruvic acid	1.33	1.36	1.53	1.38
2-Phosphoglyceric acid	1.00	1.00	1.24	1.02
3-Phosphoglyceric acid	1.00	1.00	1.00	1.00
Ribose-5-phosphate	1.28	0.88	1.13	0.79
Ribulose diphosphate	0.41	0.46	0.35	0.33
Uridinediphosphoglucose	0.83	0.37	0.74	0.35
Aspartic acid	1.88	1.17	2.22	1.27

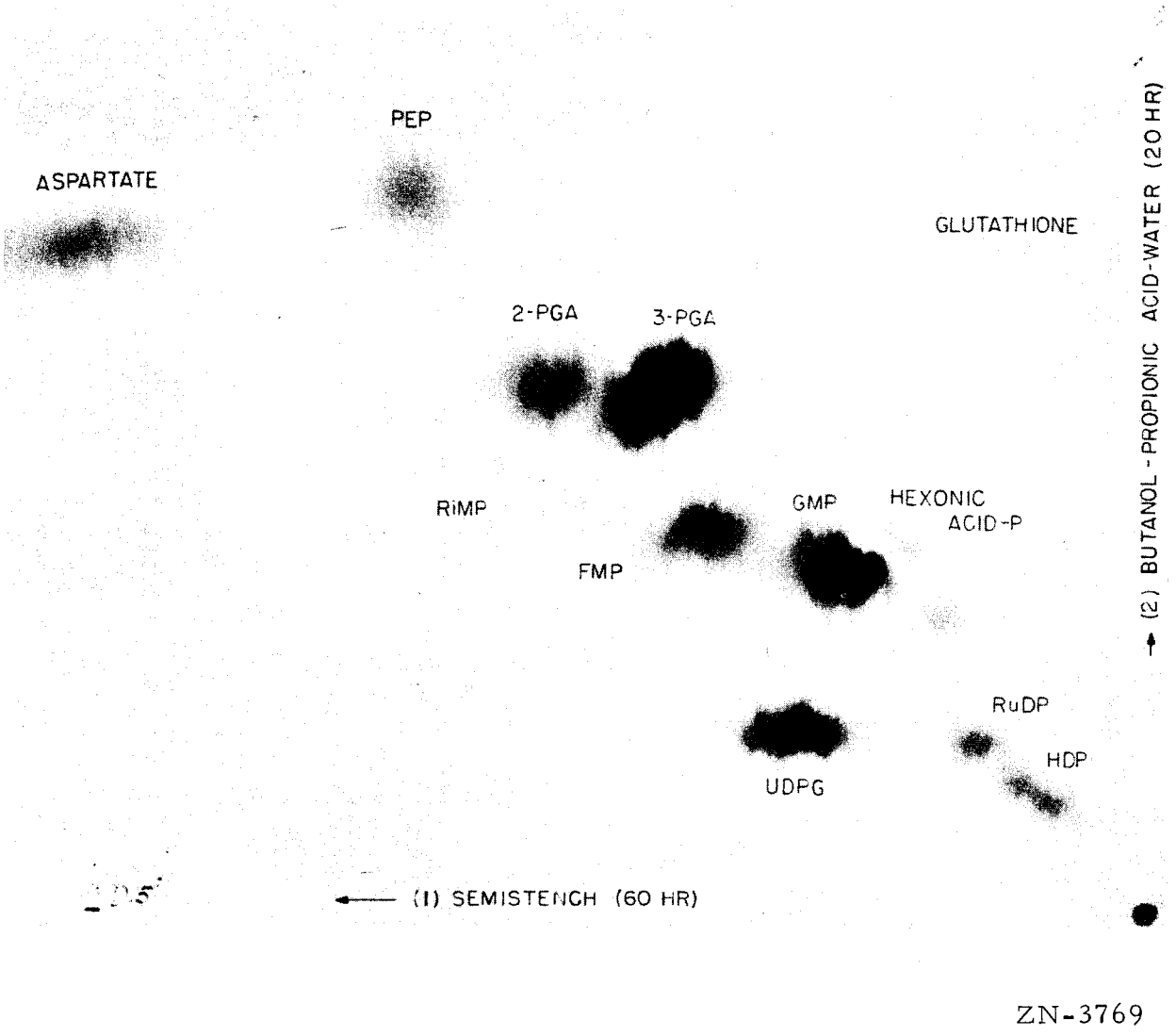

Fig. 1-3. Radioautograph of chromatogram of same extract as in Fig. 1-1, developed beyond the edges of the paper with (1) semistench (60 hr) and (2) n-butanol—propionic acid—water (20 hr). Abbreviations as for Fig. 1-1.

Table 1-III. Alteration of solvent composition during chromatogram development.
Percentage (v/v) of each component in the mixture.

1. Semistench	Water	Isopropanol	n-Propanol	n-Butanol	Isobutyric acid
Stock bottle	26.5	1.9	9.1	1.9	60.6
Chromatography trough, after development	26.2	1.8	8.5	3.2	60.4
Chromatogram, 0-7.5 cm	18.4	1.0	6.3	7.7	66.5
Chromatogram, 7.5-15 cm	16.6	1.1	6.0	7.7	68.7
Chromatogram, 15-22.5 cm	15.5	1.2	6.3	7.5	69.6
Chromatogram, 22.5-30 cm	16.5	1.2	6.6	7.4	68.3
Chromatogram, 30-37.5 cm	16.0	1.4	6.6	7.5	68.4

2. n-Butanol—propionic acid—water	Water	n-Butanol	Propionic acid
Stock bottle, fresh	30.7	46.8	22.5
Stock bottle, after 24 hr	31.5	49.0	19.5
Chromatography trough, after development (24 hr old)	25.7	50.1	24.2
Chromatogram, 0-7.5 cm	22.1	50.4	27.6
Chromatogram, 7.5-15 cm	14.9	55.4	29.6
Chromatogram, 15-22.5 cm	15.7	56.1	28.2
Chromatogram, 22.5-30 cm	16.3	57.5	26.2
Chromatogram, 30-37.5 cm	12.9	58.1	29.0

2. CHROMATOGRAPHY OF PLANT LIPIDS ON ALUMINA PAPER

Hartmut K. Lichtenthaler

In a previous paper[1] the separation of naturally occurring quinones and fat-soluble vitamins on a commercially available paper containing an alumina filler was reported. This new chromatographic technique provides rapid and ready separation of various lipid-soluble compounds tested in very short development times. It was found also to be applicable to natural extracts.

The investigation of the properties of the alumina paper towards the separation of quinones and other plant lipids has been continued by testing various additional solvents. The earlier results and the new findings are described here in greater detail. The Rf's have been established for different solvents, and these values have been checked for reproducibility. The direct application of the new technique to plant extracts as well as the limitations of the method have been examined critically.

Results and Discussion

Chromatography of pure compounds

By the procedure described earlier,[1] all known fat-soluble vitamins and various naturally occurring quinones have been separated successfully. The efficiency of the Schleicher and Schuell filter paper No. 288 for the separation of lipid-soluble quinones and vitamins is due to its content of alumina. On ordinary filter paper the quinones, vitamins, and beta-carotene are separated only poorly or not at all. Reverse-phase paper chromatographic techniques using vaseline, paraffin, or silicone as the primary phase are limited by the small amount of material that can be applied to the paper and by the impossibility of eluting the separated compounds for further analysis without simultaneous extraction of the impregnated oil. Alumina paper, however, has an obvious advantage in its higher adsorption capacity. It has the additional advantage of absorbing quinones and vitamins more strongly than beta-carotene. Moreover, the separated compounds can be eluted uncontaminated. Quinones isolated from natural extracts by this new technique showed absorption spectra in the ultraviolet region that were in good agreement with the data reported in the literature.

Cyclohexane and a mixture of cyclohexane and benzene (7:3) were found to be convenient for the separation of vitamin K_1 from β-carotene and from other quinones. This procedure also produced the purest β-carotene. For the isolation of plastoquinone 9, coenzyme Q_{10} and α-tocopherol, a mixture of cyclohexane and benzene (3:7) or pure benzene was used. α-Tocopheryl-quinone was best separated from accompanying lipids with the benzene-chloroform mixture (1:1), and for vitamin D_2 and vitamin A pure chloroform provided good separation.

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1. H. K. Lichtenthaler, in Bio-Organic Chemistry Quarterly Report, UCRL-10479, September 1962, p. 44.

The adsorption sequence on the alumina paper follows the lipid character of the compounds being chromatographed. Molecules with a high lipid solubility (e.g., hydrocarbons) are adsorbed to a much smaller extent than others with more hydrophilic groups. In the case of the quinones, one might assume that their lipid character, and consequently their order of migration, are determined by the length of the side chain in position 3. However, it will be seen that this is only partially true. Thus, plastoquinone 9, a 5,6-dimethyl-3-solanesyl-benzoquinone with 9 isoprene units (C 45) in the side chain, has a higher Rf than coenzyme Q_{10}, a 5,6-dimethoxy-2-methyl-benzoquinone with 10 isoprene units (C 50) in position 3. Thus, methoxy groups in place of methyl groups lower the Rf value considerably. The structure of the compounds discussed is represented in Fig. 2-1. α-Tocopherylquinone, a 5,6-dimethyl benzoquinone with a 3'-hydroxy phytyl side chain (C 20), when compared with PQ_9 and Q_{10} has, as may be expected, a significantly lower mobility on the chromatogram. For vitamin K_1 (2-methyl-3-phytyl naphthquinone), which has the phytyl group (as does TQ) but no hydroxyl group, one could expect a chromatographic mobility somewhat greater than that of TQ. Its Rf value, however, is even higher than those of the long-side-chain quinones PQ_9 and Q_{10}. This fact must be ascribed to the possession by the naphthoquinone ring of a lipid character apparently greater than that of benzoquinone. In spite of its phenolic hydroxyl and its short side chain of only 15 C atoms, α-tocopherol, the chromanol form of TQ, shows a higher Rf than TQ. This behavior and that of vitamin K_1 prove that double-ring structures possess a higher lipid solubility than single-ring systems. Thus, comparison of structure and Rf values makes it clear that apart from the length of the polyisoprenoid side chain, the arrangement of substituents in the quinone molecule is of essential significance for the position on the chromatogram.

Various other solvents such as isooctane, petroleum ether, carbon tetrachloride, and toluene, or mixtures thereof, were tested for separation of lipid-soluble quinones and vitamins. They also provided good separation, but had no advantages over the solvents described. More polar solvents like pyridine, methanol, and acetone were of no value, since all fat-soluble substances tested had Rf values higher than 0.9 in these solvents.

In Table 2-I the Rf values in the different solvents of quinones and vitamins, as well as some plant pigments, are listed. The values of the tested substances varied within narrow limits, especially when natural extracts were applied. There was, however, no change in the order of migration.

Normally, the walls of the chromatographic tank were lined with filter paper moistened with solvent. If this was not done, higher Rf values were obtained. In case a (Table 2-I), where the glass tank was saturated with the solvent, the solvent traveled much further from the starting point per time unit than in case b (Table 2-I), where the solvent partially evaporated from the chromatogram. Thus, in calculation of Rf's, higher values were obtained. This fact has to be taken into account when highly volatile solvents are used, and particularly when short development times are employed, as in the work discussed here (1 to 2 hr). Therefore, in all further experiments the walls were covered with filter paper in order to ensure saturation of the tank with the solvent and to obtain reproducible Rf values.

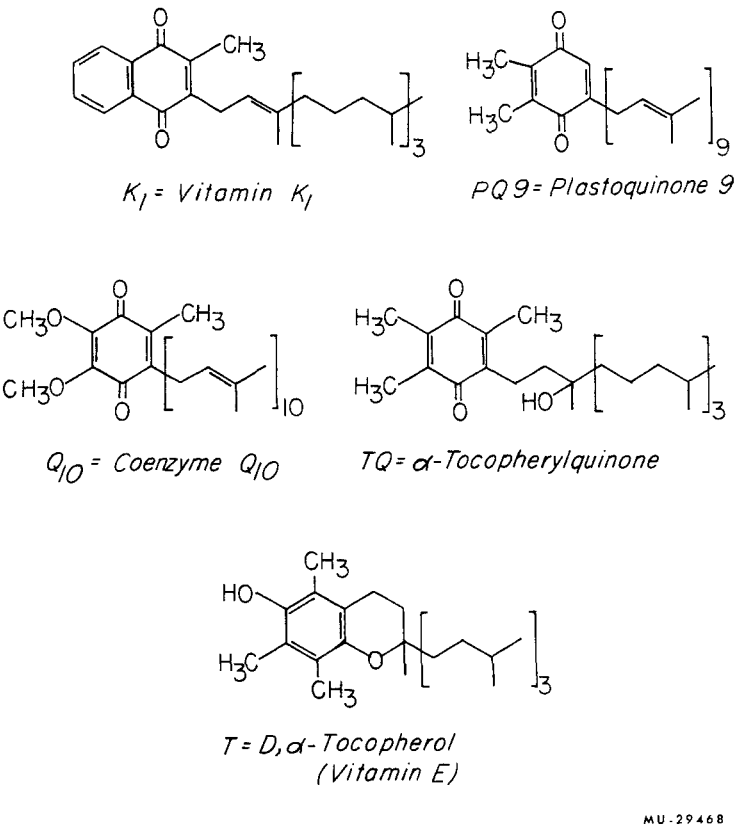

Fig. 2-1. Comparison of the structures of the quinones and vitamins from spinach leaves.

Table 2-I. Rf values for naturally occurring quinones, fat-soluble vitamins, and plant pigments on Schleicher and Schuell No. 288 paper.

Compounds	Solvents												
	Cyclohexane	Cyclohexane/benzene (7+3)		Cyclohexane/benzene (3+7)	Benzene	Benzene/$CHCl_3$ (1+1)		$CHCl_3$	Isooctane	Pet. ether (bp 30-60°)	$CHCl_4$	Toluene	
		a	b	a		a	b					a	b
β-Carotene	0.51	0.81	0.98	0.84	0.92	0.87	0.95	0.94	0.13	0.32	0.79	0.86	0.90
K_1	0.20	0.50	0.90	0.78	0.87	0.85	0.94	0.91	0.08	0.10	0.53	0.79	0.87
PQ_9	0.08	0.18	0.23	0.77	0.82	0.85	0.94	0.91	--	0.05	0.18	0.78	0.87
Q_{10}	0.05	0.09	0.16	0.47	0.70	0.83	0.90	0.91	--	--	0.13	0.55	0.85
T	--	0.06	0.13	0.26	0.46	0.48	0.64	0.82	--	--	0.10	0.32	0.78
TQ	--	--	0.05	0.05	0.14	0.41	0.50	0.73	--	--	0.05	0.09	0.65
D_2	--	--	0.05	0.08	0.13	0.40	0.41	0.59	--	--	--	0.09	0.52
A	--	--	--	0.04	0.11	0.29	0.31	0.51	--	--	--	0.09	0.38
Chl a+b	--	--	--	--	--	0.04	0.04	0.08	--	--	--	--	--
X	--	--	--	--	--	--	--	0.08	--	--	--	--	--
Ph	--	--	--	--	--	0.47	0.58	0.77	--	--	--	0.09	0.74

a. Walls of tank lined with solvent-wetted filter paper.

b. Walls of tank not lined as in (a).

Chromatography of plant extracts

The method described here has been used successfully for examining various plant extracts. It might also be employed for animal tissue extract analysis, a possibility now under investigation. β-Carotene, the principal compound accompanying and often masking quinones and vitamins in natural extracts, runs almost with the solvent front, while the chlorophylls and xanthophylls remain practically on the starting line. Thus, these normally interfering substances do not affect the separation of the other lipids.

Large amounts of extraneous fatty material in the extract, however, have a considerable influence on the Rf values of the quinones and may cause poor separation. This is particularly true for extracts from whole leaves. Therefore, purification by column chromatography should precede the separation on paper. Repeated paper chromatography is also advisable. The application of the new technique to plant extracts therefore quickly provides information as to whether quinones or tocopherols are present, before further purification and final isolation are carried out. When preliminary column chromatography is employed, the method is very useful in detecting and identifying the quinones in the various fractions eluted. Its application as a test of purity of the isolated compounds is also of great significance.

With the new method, several quinones and α-tocopherol were found in chloroplasts and quantasomes of spinach. Some of these quinones were identified as vitamin K_1, plastoquinone 9, and α-tocopherylquinone.[2] Coenzyme Q_{10}, present in the leaves of spinach, was not detected in chloroplasts and quantasomes. Further study is required for the identification of the other quinones indicated on the chromatograms. These may correspond to other plastoquinones and tocopherylquinones which recently have been shown to occur in chloroplasts.[3]

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2. H. K. Lichtenthaler, in Bio-Organic Chemistry Quarterly Report, UCRL-10479, Sept. 1962, p. 37.

3. M. D. Henninger, R. A. Dilley, and F. L. Crane, Biophys. Biochem. Res. Comm. 10, 237 (1963).

3. QUINONE AND PIGMENT COMPOSITION OF CHLOROPLASTS AND QUANTASOMES FROM SPINACEA OLERACEA

Hartmut K. Lichtenthaler

Various studies have been made on the lipid components of plants and chloroplasts. These investigations deal mostly with the isolation and determination of chlorophylls and carotenoids.[1,2] Much less attention has been paid to other lipid substances. Thus, the phosphatides and glycolipids were characterized and determined only more recently.[3] On other (mostly minor) constituents of the lipid portion our knowledge is still limited. The information on quinones and related compounds is very unsatisfactory.

In 1936 vitamin K_1 was isolated from various higher plants and determined quantitatively in a biological test.[4] This was the first natural quinone with an ubiquituous distribution in all green parts of higher plants. Other quinones, such as lapachol or lomatiol, could be isolated from plant tissues. They were, however, limited to a few species only. Dam located vitamin K_1 in chloroplasts and showed that the cytoplasm contained little or no vitamin K_1.[5] K_1, being always associated with the photosynthetic tissue, was soon considered to participate in photosynthesis. This assumption was further supported by its detection in Chlorella, the standard object of photosynthetic research, and also by studies of the relationships between the formation of chlorophyll and K_1. Plants grown in the dark do not synthesize chlorophyll and K_1. Seedlings of Picea canadensis, however, which do form chlorophyll in the dark, produced K_1 also.[6]

All these findings still did not reveal the possible site of action for K_1 in photosynthesis, but led to a great many speculations. When it was demonstrated that quinones could be substituted for dyes and ferricyanide in the Hill reaction,[7] the possible function of K_1 as an electron acceptor was discussed. In comparing the redox potentials of Hill reagents, Wessels postulated that "K_1 serves as the initial electron acceptor in photosynthesis."[8]

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1. R. Willstatter and A. Stoll, Untersuchungen uber Chlorophyll (Berlin, 1913), p. 116.
2. A. Seybold and K. Egle, Planta 26, 491 (1937).
3. J. F. G. M. Wintermans, Biochem. et Biophys. Acta 44, 49 (1960).
4. H. Dam, Schonheyder, Tage, and Hansen, Biochem. J. 30, 897 (1936); Biochem. J. 30, 1075 (1936).
5. H. Dam, E. Hjorth, and I. Kruse, Plant Physiol. 1, 379 (1948).
6. H. Dam and J. Glavind, Biochem. J. 32, 485 (1938).
7. O. Warburg and W. Luttgens, in Schwermetalle als Wirkungsgruppen von Fermentin, (Saenger, Berlin, 1947), p. 175.
8. J. S. C. Wessels, Rec. trav. Chim., Pays-Bas, 73, 529 (1954).

He suggested further that chlorophyll and K_1 may occur in a constant molecular ratio of about 200 to 1, and represents a functional group in the photosynthetic apparatus. More evidence was given for this suggestion, when it was shown that the molar ratio of chlorophyll to K_1 was relatively constant in plant families.[9] In general, 20 to 40 molecules of chlorophyll a to 1 molecule of K_1 were found in higher plants. Arnon found that K_1 derivatives such as menadione and other naphthoquinones were powerful catalysts of photosynthetic phosphorylation.[10]

When Lynch and French extracted freeze-dried chloroplasts with petroleum ether,[11] the ability of the chloroplasts to reduce dichlorophenolindophenol photochemically was lost. They attributed this change to the extraction of β-carotene, since adding a partially purified β-carotene restored the Hill activity. Bishop demonstrated that pure β-carotene had no effect on the reactivation of the Hill reaction in extracted chloroplasts.[12] He ascribed the effect to K_1, which Dam had reported to be located in chloroplasts. In analysis of petroleum ether extracts from chloroplasts, no appreciable amount of K_1 was found. This result was confirmed by Zill.[13] At this time Crane isolated plastoquinone from chloroplasts,[14] and Bishop showed that the restoration of the photochemical activity of extracted chloroplasts was due to this new quinone.[15] Whether vitamin K_1 or perhaps other quinones were present in chloroplasts was still unknown.

Park and Pon in this Laboratory isolated, from sonically ruptured chloroplasts the quantasomes, morphological subunits of grana lamellae.[16] These particles, 100 Å thick and 200 Å in diameter, contain approximately 180 chlorophyll molecules, a calculation based on the chlorophyll-to-nitrogen ratio. About one half of the weight of quantasomes consists of proteins and the other half of lipids. In an overall study of their chemical composition, an analysis of the lipids from quantasomes was started. Of particular interest were the determination of plastoquinone and the search for vitamin K_1 and other compounds that could function as electron acceptors and possibly be catalysts in the photochemical reaction of photosynthesis. It was also of importance to investigate whether the lipids are located in the same relative concentrations in the quantasomes as they are in the whole chloroplasts.

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9. H. K. Lichtenthaler, Planta 57, 731 (1962).
10. D. I. Arnon, Fed. Proc. 20, 1012 (1961).
11. V. H. Lynch and C. S. French, Arch. Biochem. 70, 382 (1957).
12. N. I. Bishop, Proc. Natl. Acad. Sci. (U. S.) 44, 501 (1958).
13. L. P Zill and E A. Harmon, Biochim. et Biophys. Acta 57, 573 (1962).
14. F. L. Crane, Plant Physiol. 34, 128 (1959).
15. N. I. Bishop, Proc. Natl. Acad. Sci. (U. S.) 45, 1696 (1959).
16. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1961).

Experimental Procedures

Isolation of chloroplasts

Chloroplasts were isolated by a slight variation of the procedure described by Park and Pon.[16] Spinach, obtained from a local market, was washed with tap water, midribs were removed, and the remainder of the leaves (500 g) homogenized for 30 sec in 1000 ml sucrose (0.5 M)–potassium phosphate (0.1 M) buffer adjusted to pH 7.0. After filtration through eight layers of cheesecloth, cell debris was removed from the filtrate by centrifuging at 200×g for 5 min. The supernatant was then centrifuged at 1000×g for 15 min to precipitate the chloroplasts. The latter were suspended in the same buffer and centrifuged at 500×g for 15 min. The chloroplast precipitate was resuspended in potassium phosphate buffer of pH 7.0. This procedure resulted in a considerable loss of chloroplasts. However, rigorous purification was deemed necessary to ensure a pure chloroplast fraction not contaminated by other cell particles, such as mitochondria, which contain coenzyme Q_{10} and perhaps other quinones. Centrifugation of the resuspended 1000×g precipitate at 500×g gave a pure chloroplast fraction free of any interfering contaminations.

Isolation of quantasomes

The chloroplast suspension was sonicated in a Raytheon sonicator for 90 sec. The ruptured chloroplasts were centrifuged at 20,000×g (10 min) and then at 145,000×g (30 min). The last centrifugation gave three principal fractions: A green precipitate (aggregates of 5 to 6 quantasomes), a colorless supernatant, and a layer of lipid-containing material, which rises to the surface of the tube during centrifugation. The quantasome fraction was suspended in phosphate buffer of pH 7. All operations during isolation of chloroplasts and quantasomes were carried out near or at 0° C. When the chloroplast or quantasome suspensions were not used immediately after isolation, they were kept in the dark and in the cold room near 0° C until further treatment.

Extraction of lipids

Chloroplast or quantasome suspensions containing approximately 100 mg of chlorophyll were slowly poured with continuous shaking into 500 ml aqueous acetone, so that the final acetone concentration was 80%. The extraction scheme is outlined in Fig. 3-1. The denatured protein was removed by centrifugation at 500×g for 5 min. The supernatant chlorophyll extract was collected and an aliquot was taken for the chlorophyll determination. The extraction of lipids with acetone was incomplete despite the almost quantitative extraction of chlorophylls. The grey residue therefore was further extracted with 100 ml acetone, 200 ml of a mixture of acetone-isooctane (1:1), and 200 ml pure isooctane. The combined extracts were placed in a separatory funnel, a solution of saturated sodium chloride was added, and by slight shaking all lipid-soluble compounds were dissolved in the epiphase. The hypophase was re-extracted twice with isooctane to leave a colorless solution.

Treatment with saturated sodium chloride solution prevented the formation of emulsions on the interphase, which usually occurs when pure water is used instead. Also, a quantitative extraction of the hypophase was accomplished. When occasionally emulsions were formed, they were broken by centrifugation at 1000×g for 5 min. The isooctane extract was dried with

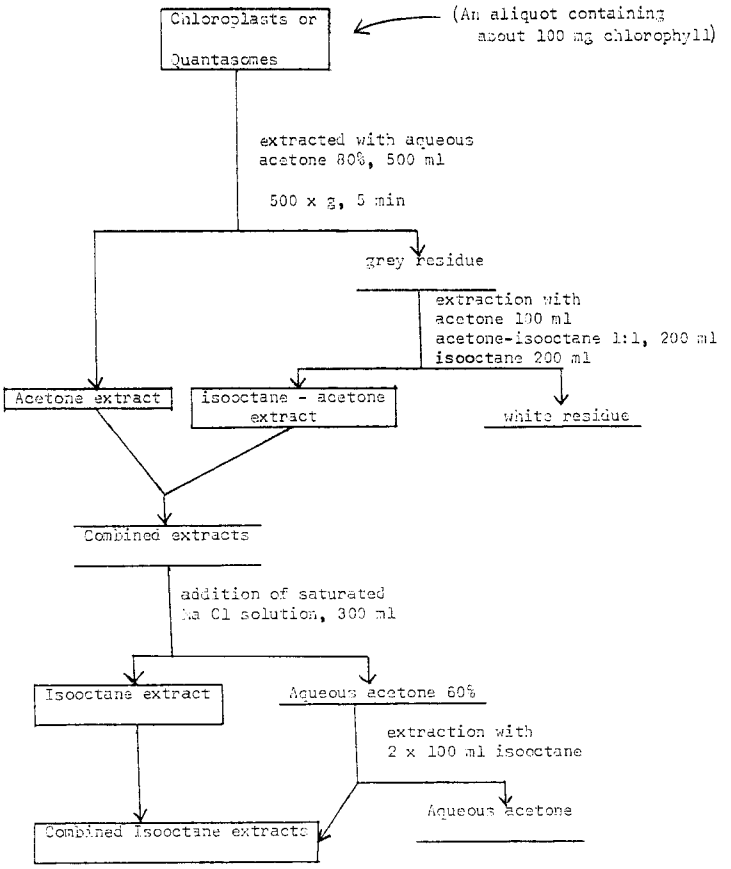

Fig. 3-1. Diagram of chloroplast and quantasome extraction.

anhydrous sodium sulfate, concentrated in vacuo to a smaller volume, and stored under argon in the dark and in the deep freeze for further purification. To avoid destruction of quinones, all operations during extraction were carried out in dim light.

A small aliquot of the extract was chromatographed on alumina paper by a technique described elsewhere.[17] This test quickly gave information on the nature of quinones and tocopherols in the extract.

Column chromatography of isooctane extract

The extract was chromatographed on a column packed with aluminum oxide (Ignited Powder, manufactured by Baker and Adamson). The dimensions of the column were 6 cm (diameter) and 25 cm (height). Adsorbent height was about 15 cm. The column was moistened with 200 ml of isooctane and then the extract was placed on the column and eluted with isooctane. A slight suction (vacuum line) was employed. β-Carotene came through with 500 ml isooctane in about 8 min. Further elution was carried out with 600 ml of peroxide-free diethyl ether. Subsequently, several yellow bands were eluted with 1 liter of a mixture of diethyl ether–acetone (1:1). By further development with the same mixture the chlorophylls were eluted. The total elution time varied between 20 and 30 min. The three fractions were concentrated in vacuo and made up to known volumes in calibrated flasks. Then they were placed in the dark in the deep freeze until further purification.

Small samples of each fraction were analyzed by chromatography, by use of the method reported earlier.[17] The fractions contained the following compounds:

Fraction I: β-carotene, quinones, and α-tocopherol.

Fraction II: traces of β-carotene, quinones, and phaeophytin.

Fraction III: xanthophylls, mainly lutein, violaxanthin, and neoxanthin.

Thus, the rapid paper chromatographic test gave good information about the distribution of the lipids in the different fractions. This knowledge was of great importance for the further purification and isolation of different compounds.

Quinones and pigments can be separated on an alumina column in purer fractions by choosing less polar solvents and by avoiding suction. This, however, results in longer elution times. Since quinones, particularly plastoquinone 9, are partially destroyed when in contact with alumina for a few hours, short development times were required. The average time for elution of the three fractions indicated above was between 20 and 30 min. The chromatography of the extracts on the alumina column provides complete and rapid separation of quinones and carotenoids from chlorophylls, which would interfere with the determination of the other lipids.

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17. H. K. Lichtenthaler, Paper 2 in this report.

Chromatography on polyethylene powder

Another very useful adsorbent for the separation of plant lipids, and particularly for the isolation of quinones, is polyethylene powder. Linear polyethylene powder, melt index 0.9, was obtained through the courtesy of the Dow Chemical Co., Midland, Michigan. The development on this column, however, is considerably longer and the adsorption capacity is much less than that of alumina. Thus, for the separation of larger quantities of extracts, columns with larger dimensions have to be used. Since bigger columns did not provide a satisfactory separation, the extract had to be divided and chromatographed on two columns.

The extract in 70% aqueous acetone was placed on a column that was first washed with 70% aqueous acetone. The chromatogram was developed with 70% aqueous acetone and the colored zones were successively eluted. First, several yellow bands moved down. They contained carotenoids, mainly violaxanthin, neoxanthin, and lutein. With 80% acetone, the chlorophylls were removed from the column, leaving the carotenes, quinones, and phaeophytin behind. This pigment zone was cut from the column and extracted with peroxide-free diethyl ether. The extracts were concentrated and analyzed by paper chromatography for their quinone content, as reported before. The quinones and α-tocopherol were found in the β-carotene and phaeophytin fraction.

The xanthophyll fraction was transferred from acetone into isooctane, following the procedure described in Fig. 3-1. For the separation of the xanthophylls from each other, the extract was chromatographed on Schleicher and Schuell paper No. 288, with 5% isopropanol in petroleum ether as solvent, using the circular technique. The Rf's were 0.7 for lutein, 0.4 for violaxanthin, and 0.15 for neoxanthin. The carotenoids could be eluted from the paper with diethyl ether. The spectra taken in the visible and ultraviolet regions were in good agreement with the data reported in the literature.

Quantitative determination of quinones and pigments

Chlorophylls: The chlorophyll content was determined with light-absorption measurement in 80% aqueous acetone at 663 and 645 mμ or at 652 mμ for a rough check according to the method of MacKinney.[18] Chlorophyll a and b were calculated separately.

Xanthophylls: Lutein was determined by measuring the optical density in hexane at 447 mμ, violaxanthin in hexane at 438 mμ, and neoxanthin in hexane at 433 mμ. Extinction values are reported by Goodwin,[19] but if these were unknown in hexane, the coefficient for β-carotene was used.

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18. G. MacKinney, J. Biol. Chem. 140, 315 (1941).

19. T. W. Goodwin, in Modern Methods of Plant Analysis, Vol. III, K. Paech and M. V. Tracey, Eds. (Springer-Verlag, Berlin, 1955).

β-Carotene: The β-carotene content was determined by measuring the optical density in hexane at 450 mμ, using the molar extinction coefficient from Zechmeister.[20]

Quinones and tocopherol: From the quinone-containing fractions, quinones were isolated by paper chromatography on Schleicher and Schuell paper No. 288, using the solvents described earlier.[17] The quinones and α-tocopherol were eluted with diethyl ether, the ether evaporated in vacuo, and the compounds taken up in the special solvents for which the specific absorption coefficients were known. These were hexane for vitamin K_1,[21] ethanol for α-tocopheryl-quinone and α-tocopherol,[22] and ethanol for plastoquinone A and B.[23] The concentrations of the substances listed here were calculated from the optical density of the measured solution according to the values described in the literature.

Results and Discussion

With the techniques described in the experimental section, pigments and quinones from whole chloroplasts and chloroplast lamellae (quantasomes) were isolated and determined quantitatively. The values obtained are shown in Table 3-I in terms of amount per 100 mg total cholrophyll. There is only little variation in the pigment concentration in different experiments. The values for quinones, however, varied more, possibly because the procedure for their isolation was not so simple as for the pigments.

The pigment and quinone concentrations for quantasomes were the same as for chloroplasts or only slightly less. This observation may lead to the conclusion that these pigments and the quinones are located in the quantasomes, as has been shown for chlorophyll,[16] and are not associated with the soluble proteins containing the enzymes of the carbon-reduction cycle.

When the quantasomes are centrifuged down at 145,000 g, a yellow zone rises to the surface of the tube. When this yellow layer is analyzed, β-carotene, all chloroplast quinones, and α-tocopherol are found. Only traces of chlorophylls and xanthophylls were detected after centrifugation had been carried out for at least 30 min. In relation to quinones, there was less β-carotene present than in chloroplasts or quantasomes.

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20. L. Zechmeister, Chem. Rev. 34, 267 (1934).

21. D. I. Ewing, I. M. Vanderbelt, and O. Kamm, J. Biol. Chem. 131, 345 (1939).

22. F. J. Mulder and K. J. Keuning, Rec. Trav. Chim. 80, 1029 (1961).

23. L. P. Kegel, M. D. Henninger, and F. L. Crane, Biochem. Biophys. Res. Comm. 8, 294 (1962).

Table 3-I. Relative amounts (by weight) of quinones and pigments in chloroplasts and quantasomes. The values for quinones are given: (a) after elution from the paper chromatogram and (b) after further chromatographic purification (see text). X = total xanthophylls.

	Chloroplasts				Quantasomes		
	(a)	(b)	(a)	(b)	(a)	(a)	(b)
Chlorophyll a + b	100		100		100	100	
Chlorophyll a	70.3		71.5		70.3	70	
Chlorophyll b	29.7		28.5		29.7	30	
β-Carotene (c)	3.5		4.15		3.67	3.4	
Lutein	6.28		5.95		5.85	5.6	
Violaxanthin } X	1.82		1.89		1.78	1.6	
Neoxanthin	1.65		1.66		1.5	1.6	
Plastoquinone A	6.45	3.8	5.5	4.1	6.3	4.8	3.1
Plastoquinone B	5.3	3.47	3.7	2.8	--	4.4	2.4
Vitamin K_1	0.88	0.84	--	0.8	0.96	0.8	0.74
α-Tocopherol	1.91	1.48	2.0	1.3	1.65	--	1.3
α-Tocopherylquinone	0.98	1.16	0.87	1.0	--	0.7	0.8
a/b	2.36		2.41		2.36	2.37	
x/c	2.80		2.30		2.50	2.62	
$\frac{a+b}{x+c}$	7.50		7.33		7.80	8.15	

Electron micrographs of the lipid layer showed that it contained large osmiophilic granules which were not associated with the carbon dioxide fixation.[16] These granules might consist of lipids such as diglycerol phosphate.[24] Whether the granules have a particulate structure is not known. In electron micrographs they appear as spherical objects with an average dimension of 140 mμ. The size, however, is not constant, and varies between 110 and 170 mμ, as may be seen on Plate XII from Park and Pon.[16]

Treatment with hexane removes only part of the lipids, and leads to the formation of emulsions that must be broken by centrifugation. The osmiophilic granules can be extracted only with more polar solvents, such as ethanol

24. A. A. Benson, J. F. G. M. Wintermans, and R. Wiser, Plant Physiol. 34, 315 (1959).

and acetone. The residue obtained is white and consists of denaturated protein. This observation might indicate that the granules contain both lipids and protein. It would also suggest that the lipids are bound in some way to that protein. Therefore it seems that the osmiophilic granules do not consist merely of large drops of fatty material in which fat-soluble quinones from chloroplast lamellae are dissolved.

When chloroplasts are sonicated during the isolation of quantasomes, the granules are not broken but keep their form. Since they contain lipids and are thus water-insoluble, they rise to the surface of the aqueous buffer solution in a way similar to fat drops in water. When the pigmented quantasomes are centrifuged at 145,000 × g, this granule fraction is revealed on the surface.

A yellow lipid layer containing also quinones, α-tocopherol, and β-carotene was found on the surface of chloroplast suspensions that had stood for several days. It is suggested that this lipid layer also consists of osmiophilic granules. The chloroplasts settle down slowly, leaving an almost colorless supernatant. It is very likely that during storage the chloroplast structure gets disorganized. The chloroplast membrane is partially opened, thus releasing the granules. This also indicates that neither the grana lamellae nor the stroma protein holds the granules. Thus, they are not bound--or are only loosely bound--to the internal chloroplast structure and are held in the chloroplast only as long as the chloroplast membrane is intact. It is likely that the protein in the yellow lipid layer is part of the granule and that the granule itself is not bound or is only loosely bound to the chloroplast structure.

If the granules are particles, their function in photosynthesis is not yet known. Their quinone content would suggest an auxiliary role in the electron transport of photosynthesis. If it can be proved that the granules possess a structure and are real particles, the term "osmiophilic granule," which is derived from their behavior in electron micrography, would be too unspecific. According to their chemical composition, a name such as "liposome" would be more reasonable.

In comparison with the quantity of quantasomes at the bottom of the centrifuge tube, the lipid layer was small. Since quantasomes contain essentially the same quinone concentration as chloroplasts, it seems clear that most of the quinones are located in the chlorophyll lamellae and few are associated with the osmiophilic granules of the lipid layer.

When this investigation was started, the question of whether or not vitamin K_1 was present in chloroplasts was still open. With the paper chromatographic technique described, K_1 could be detected in both chloroplasts and quantasomes. It was identified by its uv spectrum, by the Rf values in different solvent systems, by cochromatography, and also by the color reactions with sodium ethylate and xanthanhydride. The presence of K_1 in chloroplasts was confirmed in an independent work by Kegel and Crane.[25] However, the amount found by that group was one molecule of K_1 for 100 molecules of total chlorophyll, a value obtained also by Dam[5] in a biological test. The content of K_1 measured in this Laboratory is 1.5 to 2.0 for 100 molecules chlorophyll. This is in good agreement with earlier findings using another method.[9]

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25. L. P. Kegel and F. L. Crane, Nature 194, 1282 (1962).

The variation in the K_1 content may be ascribed to the different spinach samples investigated. In an earlier publication it was shown that the K_1 content of higher plants depends on the age of the plant tissue and also on the light intensity in which the plants were grown. The K_1 content reaches a maximum only at the end of the vegetation period, and is substantially higher in sun-illuminated leaves than in leaves grown in shade. A variation in the K_1 content in different samples was therefore to be expected.

Since in spinach leaves the same amounts of K_1 were found as in chloroplasts, one can assume that the total K_1 is located in chloroplasts. This finding confirms earlier results of Dam,[5] which were obtained by testing the vitamin K_1 activity of chloroplasts and cytoplasm in a biological assay.

Plastoquinone A and B were detected in both chloroplasts and quantasomes. The amounts for PQA, however, were less than those reported by Kegel, Henninger, and Crane.[23] On a molar basis they found for PQA 16 moles per 100 moles of chlorophyll, whereas in our own experiments only 8 moles PQA per 100 moles of chlorophyll could be detected. This difference is very likely due to the different extraction and isolation methods. In the case reported here the quinones were separated on paper after preliminary column chromatography. The quinone solutions obtained by elution of the quinone bands from the paper apparently were purer than the fractions that Kegel[23] et al., eluted from columns.

The spectra of the bands eluted from paper showed the absorption maxima at 255 mμ, but still contained small impurities. After further purification on paper, highly pure quinone solutions were obtained. Reduction with potassium borohydride decreased the absorption at 255 mμ to give a new peak at about 286 mμ. The quantitative values were calculated from oxidation-reduction changes at 255 mμ: (a) after the first separation on paper and (b) after further purification by paper chromatography. That smaller quantities of plastoquinones were found in (b) (Table 3-I) may be due either to other compounds also contributing to the measurement in (a) or to a loss of PQ occurring during the purification that gave (b). Since oxidation-reduction changes are good criteria for the quantitative determination of the PQ's even in somewhat impure solutions, it is likely that the lower values in (b) must be ascribed to losses during purification. This is also supported by the observation that other more stable quinones (K_1 and TQ) gave after purification almost unchanged values.

For plastoquinone B, which has a mobility lower than PQA on the paper chromatogram, about 4 moles per 100 moles chlorophyll was found. We could not detect as high amounts (8 moles) as were reported by Kegel et al.[23] The amounts of both PQA and PQB in spinach chloroplasts were only half of the ones given by those authors. We suspect therefore that they measured highly impure plastoquinone fractions. The impurities then indicated gave far too high quinone concentrations in the quantitative determination by uv spectroscopy. The variability of the published values is demonstrated by comparing the Kegel's values[23,25] with those in a recent publication.[26] There are considerable differences in the values given in a and b of Table 3-II.

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26. M. D. Henninger, R. A. Dilley, and F. L. Crane, Biochem. Biophys. Res. Comm. 3, 237 (1963).

Table 3-II. Comparison of the quinone values given by Crane and co-workers in two different publications, a (Ref. 23) and b (Ref. 26) with those of our own investigations, c. The quinone concentration is given in moles per 100 moles of chlorophyll. The values in b originally reported in mg per mg of chlorophyll were calculated by us for a molar basis.

	a.	b.	c.
PQA	16	8	8
PQB	8	≈ 1.5	4
PQC	10	≈ 1.6	-
K_1	0.8	1.6	2
α-TQ	--	1	2

The newest quinone concentration given by Henninger et al.[26] is in good agreement with our own data. The authors suggest, however, that their new values (b) are somewhat low.

By all methods of investigation employed until now in this Laboratory no plastoquinone C could be detected in either chloroplasts or leaves of spinach. Its concentration has been reported to be 1 mole for 10 moles of chlorophyll.[23] The extinction was given as $E^{1\%}_{1cm} = 62$. Assuming that the molar extinction coefficient is the same for all plastoquinones (about 19,000), an approximate molecular weight can be calculated for PQC, which would be 2,780 as against 780 for PQA. Since PQC was said to be a homologue of PQA, the difference in the molecular weight can only be ascribed to more isoprene units in the side chain at position 3. This would indicate a polyisoprenoid chain of great length. This is very unlikely, since no substance with more than 10 isoprene units in one chain has been isolated from natural material. Moreover, the chromatographic behavior on columns and the Rf value on paper given for this substance would suggest a considerably less lipid-soluble compound with far fewer isoprene units than PQA. It is therefore assumed that the quinone isolated by Kegel et al.[23] was highly impure and might not have had the structure of a plastoquinone. This assumption is further supported by the detection of α-tocopherylquinone in chloroplasts,[27] a quinone with properties similar to those of the reported "PQC." The absorption maxima in ethanol are at 261 and 269 mμ. Reduction decreases the light absorption in the maxima and gives a small new peak at 286 mμ (Fig. 3-2). It also is a brown oil and has the same Rf value as the presumed "PQC." It is therefore suggested that "PQC" isolated by Kegel, Henninger, and Crane[23] was a highly contaminated TQ.

27. H. K. Lichtenthaler, in Bio-Organic Chemistry Quarterly Report, UCRL-10479, Sept. 1962, pp. 37 and 44.

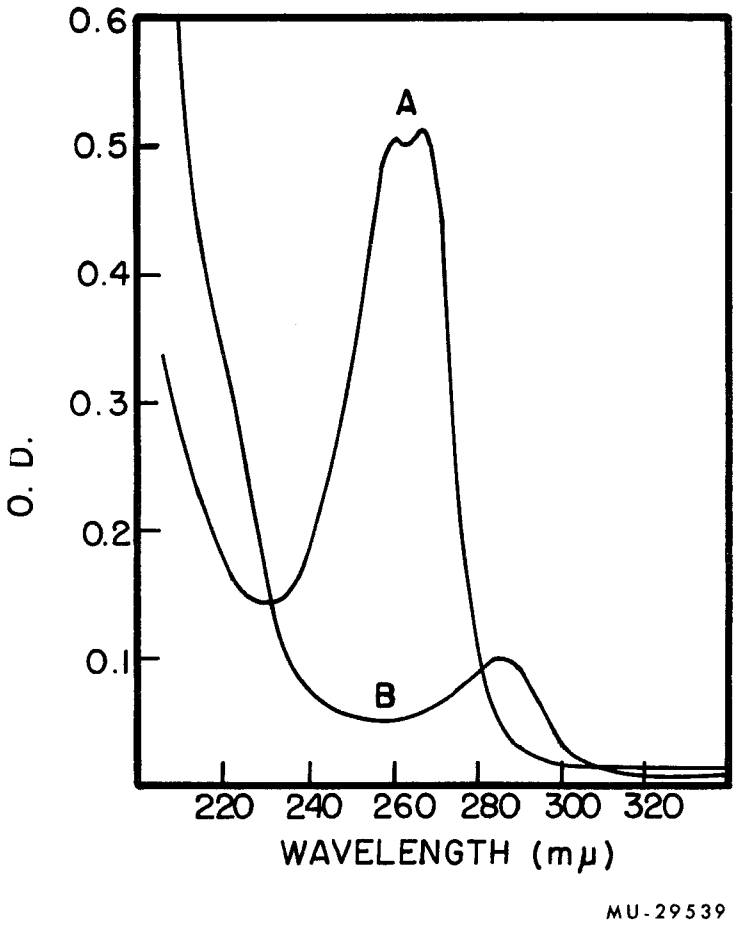

Fig. 3-2. Absorption spectrum of α-tocopherylquinone, isolated from spinach chloroplasts (in absolute ethanol). A, oxidized form; B, reduced with potassium borohydride. The isobestic points for oxidized and reduced form are at 231, 281, and 306 mμ.

α-Tocopherylquinone (TQ) was isolated from chloroplasts and quantasomes in a concentration of 2 moles for 100 moles chlorophyll. Its mobility on the alumina column and on Schleicher and Schuell paper No. 288 is a little lower than that of phaeophytin. Thus, phaeophytin indicates the position of TQ on chromatograms. TQ was detected not only in acetone extracts from chloroplasts, but also when unextracted quantasomes were applied to the paper. This indicates that TQ is not formed during extraction or isolation procedures from α-tocopherol by oxidation. Recently Henninger, Dilley, and Crane[26] confirmed the presence of TQ in chloroplasts. However, they found only 1 mole of TQ for 100 moles of chlorophyll.

α-Tocopherol was found in a concentration of 3 to 4 moles per 100 moles of chlorophyll. Identification was carried out by the Emmerie-Engel test[28] cochromatography, and two-dimensional chromatography on zinc carbonate paper, using the method described by Green, Marcinkiewicz, and Watt.[29] Upon oxidation of α-tocopherol with ceric sulfate or gold^{+3} chloride, α-TQ was obtained.

The presence of α-tocopherylquinone and α-tocopherol, which is the chromanol form of α-TQ, contributes more evidence on some of the theories of the functional form of quinones in photosynthesis. Quinones have been discussed as the initial acceptors of phosphate in photosynthetic phosphorylation. A proposed reaction mechanism would require the chromanol form, which could transfer phosphate to ADP.[30]

The carotenoids of spinach chloroplasts were recently determined to be lutein, violaxanthin, neoxanthin, and β-carotene.[31] Their values are compared with our own results in Table 3-III. For β-carotene and neoxanthin, the measured concentrations are in good agreement. The lutein content, however, was found to be higher and the violaxanthin content lower than reported in b, Table 3-II.

The differences are too large to be explained by the use of different extinction coefficients. However, there are physiological variations in the carotenoid content of spinach. Under intense light in N_2, a decrease in the violaxanthin concentration is observed, accompanied by a nearly stoichiometric increase in zeaxanthin.[32] This indicates that there are transitions between violaxanthin and zeaxanthin. Depending on the conditions used, concentrations of the lutein-zeanthin fraction varied from 50 to 39% of total carotenoids, while those of violaxanthin varied inversely from 8 to 19%.[32] The total amount of carotenoids did not change, however.

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28. A. Emmerie and C. Engel, Rec. Trav. Chim. 57, 1351 (1938).

29. J. Green, S. Marcinkiewicz, and P. R. Watt, J. Sci. Food Agric. 6, 274 (1955).

30. V. M. Clark and A. Todd, in Ciba Symposium on Quinones in Electron Transport (J. and A. Churchill, London, 1961) p. 190.

31. H. Y. Yamamoto, C. O. Chichester, and T. O. M. Nakayama, Photochem. Photobiol. 1, 53 (1962).

32. H. Y. Yamamoto, T. O. M. Nakayama, and C. O. Chichester, Arch. Biochem. Biophys. 97, 168 (1962).

Table 3-III. Carotenoid components as percentages of total carotenoid content from spinach chloroplasts.

a. Average values from Table 3-I.
b. Data taken from Ref. 29.

	a	b
β-Carotene	28.5	28
Lutein (including also zeaxanthin)	45.5	42
Violaxanthin	13.7	17.5
Neoxanthin	12.3	12.5
Total	100.0	100.0

With the simultaneous determination of chlorophylls (a + b), xanthophylls (x), and beta-carotene (c) accomplished, it was now possible to calculate for spinach the weight ratios for a/b, x/c, and $\frac{a+b}{x+c}$. These data are given in Table 3-I. The average values were a/b = 2.38, x/c = 2.56, and $\frac{a+b}{x+c}$ = 7.75. There were no significant differences in these numbers between chloroplasts and quantasomes, although the ratio of green to yellow pigments in the case of quantasomes is somewhat higher, thus indicating less carotenoids. The data available, however, are still too few to allow a final conclusion.

On a molar basis the ratio of green to yellow pigments was found to average 4.55. Seybold and Egle reported for higher plants the ratio 2.4 to 3.7,[2] and Willstätter and Stoll 2.8 to 6.0.[1] These authors demonstrated that higher values (less carotenoids) were obtained in shadow leaves which were not fully exposed to the sunlight. The ratio of 4.55 for spinach is relatively high and might indicate that not all spinach leaves were exposed to the light, probably because of growing too thickly. The value for x/c was 2.56, and is in better agreement with the data given by Willstätter and Stoll (1.5 to 2) than with those of Seybold and Egle (4.5 to 5.1).

The molar distribution of quinones and pigments is represented in Table 3-IV. In each case, the number of moles per 100 moles chlorophyll is given. About 22 moles of carotenoids and about 20 moles of quinones were found. That the quinone compounds contribute as much to the lipid components of chloroplasts on a molecular and on a weight basis as total carotenoids is considered very significant. The distributions of quinones and pigments in chloroplast lamellae, however, are not constant, and the values given here may vary depending on the physiological conditions. Particularly the age of the tissue and the light intensity, in which the plants are grown, influence the pigment and quinone concentration considerably.

For some time the function of carotenoids in photosynthesis as possible exciton donors or oxygen carriers has been studied without satisfactory results.

Table 3-IV. Relative molar concentration of quinones and pigments in chloroplasts and quantasomes.

	Chloroplasts		Quantasomes		Average	Moles
Chlorophyll a+b	100	100	100	100	100	100
Chlorophyll a	70	72	70	70	70	
Chlorophyll b	30	28	30	30	30	
β-Carotene	6	7.1	6.3	5.8	6.3	approx 22
Lutein	10.2	9.7	9.5	9.1	9.6	
Violaxanthin	2.8	2.9	2.7	2.5	2.8	
Neoxanthin	2.6	2.5	2.4	2.6	2.5	
Plastoquinone A	8	6.8	7.8	6.1	7.2	approx 20
Plastoquinone B	5	3.5	--	4.2	4.2	
Vitamin K_1	1.8	1.5	2.0	1.8	1.8	
α-Tocopherol	4.1	4.3	3.5	2.7	3.7	
α-Tocopherylquinone	2.4	1.8	--	1.6	1.9	

It is obvious that quinones, which may react in the oxidized, reduced, or chromanol form, are better catalysts for the photochemical reaction than carotenoids.

It has been shown in several investigations that all quinone compounds reported here stimulate the Hill reaction.[26, 33] It has also been postulated that hydroquinone or chromanol phosphates may be the precursors for ATP formation during photophosphorylation. However, much more research has to be done to understand completely the quinone function.

Since it was shown that quantum conversion and electron transport are carried out by aggregates of 5 to 6 lamellar subunits (quantasomes), which contain the total chlorophyll, it was necessary to determine the other quantasome pigments and quinones that might stabilize the system or act as catalysts. It is hoped that the data on the distribution of carotenoids and quinones in chloroplast lamellae presented in this paper will contribute to a better understanding of photosynthesis.

33. D. W. Krogmann and E. Olivero, J. Biol. Chem. 237, 3292 (1962).

4. THE LIPID COMPOSITION OF CHLOROPLAST LAMELLAE FROM SPINACEA OLERACEA

Hartmut K. Lichtenthaler and Roderic B. Park

Much research in photosynthesis today is concentrated on the primary quantum conversion process and subsequent electron transport leading to the formation of ATP and reduced TPN. Park and Pon showed that quantum conversion and the electron transport reactions are carried out by the lamellar structures of spinach chloroplasts.[1]

Furthermore, the intact lamellae are not necessary to perform these processes. Aggregates of five or six lamellar subunits (quantasomes) are fully active in quantum conversion and electron transport. Quantasomes are morphologically defined particles (100 to 200 Å). They contain the photosynthetic pigments (chlorophylls and carotenoids) and also several quinones and α-tocopherol.[2] Since quantasomes may represent the smallest functional photosynthetic unit able to carry out quantum conversion and electron transport, it is desirable to obtain a more complete chemical and physical picture of these particles.

Lipids and proteins in about equal parts make up the quantasome structure. Pigments constitute approx 25% of the lipid material. Various other lipids are reported to be associated with the photosynthetic tissue, and they account for some of the remaining chloroplast lipids. These reports, however, are widespread in the literature and often presented without recognizing the relations to the various other lipids.

In this paper we summarize the information on lipids in spinach chloroplasts and calculate the distribution of lipid substances in chloroplast lamellae, on the basis of a minimum molecular weight of 960,000 per atom of manganese.

Chloroplasts contain both the light and dark reactions of photosynthesis, and consist of pigments, lipids, soluble and insoluble protein, and water. A number of papers have reported the total amount of lipids, protein, and pigments in leaves and chloroplasts of various higher plants. Some of the data given for chloroplasts of Spinacea oleracea are as follows.

Menke found that 30.9% of the dry weight of chloroplasts were lipids, the rest being protein and other substances.[3] He mentioned that most of the leaf lipids are located in the chloroplast. Menke specified that the ether-soluble lipids are a mixture of fatty acids--glycerides, phosphatides, and

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1. R. B. Park and N. G. Pon, J. Mol. Biol. 3, 1 (1962).
2. H. K. Lichtenthaler, this report, paper 3.
3. W. Menke, Z. Botan. 32, 273 (1938); Z. Physiol. Chem. 257, 43 (1938).

pigments. In acetone extracts 80% of the total chloroplast lipids were present and consisted of pigments, triglycerides, and sterols.[4] The acetone-insoluble fraction contained phosphatides and waxes. In the pigment-free acetone fraction the lipid distribution was glycerol, 5.1%; fatty acids, 42.2%; triglycerides, 49%, and sterols, 2.1%.

The fatty acids were given by Speer, Wise, and Hart,[5] with linoleic acid, 34.7%, and linolenic acid, 12.7%, as the main components for spinach leaves; 53% of the fatty acids occurred in the free form and 47% as glycerides. Wolf, Coniglio, and Davis[6] give the composition of total fatty acids, free and bound ones, as linolenic acid 68.9%; an unidentified acid with C_{16} and 3 double bonds, 10.8%; palmitic acid 11.2%; linoleic acid 4.6%; and traces of others.

Wiser and Benson reported the presence of a sulfolipid in chloroplasts.[7] Wintermans provided values for phospholipids, a total of 53 moles of phospholipids per 105 moles of chlorophyll.[8] Zill and Harmon reported that pigments, phospholipids, glycolipids, diglyceride, and digalactosyl glycolipid make up 86% of the total chloroplast lipids.[9] The rest consisted of waxes and hydrocarbons (2%), lecithin, and other minor constituents.

The concentrations of quinones were determined by Kegel, Henninger, and Crane,[10] and Henninger, Dilley, and Crane.[11] The distribution of quinones and carotenoids in relation to the chlorophylls for chloroplast lamellae was estimated in this Laboratory.[2]

From all the data now available it was possible to calculate the relative concentrations of lipid components in chloroplast lamellae. Since manganese is present in chloroplast lamellae, and is required for oxygen evolution in photosynthesis, Park and Pon calculated a minimum molecular weight of 9.6×10^5 for a photosynthetic unit, based on 1 manganese atom.[12] The calculation of the quantasome mass, a morphological 200×100-Å subunit of chloroplast lamellae, from density and volume measurements, yielded a molecular weight between 1 and 2 times the minimum molecular weight given above.

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4. W. Menke and E. Jacob, Z. Physiol. Chem. 272, 227 (1942).
5. J. H. Speer, E. C. Wise, and M. C. Hart, J. Biol. Chem. 82, 105 (1929).
6. F. T. Wolf, J. G. Coniglio, and J. T. Davis, Plant Physiol. 37, 83 (1962).
7. A. A. Benson, J. F. G. M. Wintermans, and R. Wiser, Plant Physiol. 34, 315 (1959).
8. J. F. G. M. Wintermans, Biochim. Biophys. Acta 44, 49 (1960).
9. L. P. Zill and E. A. Harmon, Biochim. Biophys. Acta 57, 573 (1962).
10. L. P. Kegel, M. D. Henninger, and F. L. Crane, Biochem. Biophys. Res. Comm. 8, 294 (1962).
11. M. D. Henninger, R. A. Dilley, and F. L. Crane, Biochem. Biophys. Res. Comm. 10, 237 (1963).
12. R. B. Park and N. G. Pon, J. Mol. Biol., in press.

Park and Pon reported that lipids make up 52% of the chloroplast lamellae, while the remaining 48% is protein. Thus, lipid and protein contribute to the minimum molecular weight 495,000 and 465,000, respectively.

In the display on the following page are presented the lipid and protein portions of the lamellae. The lipid portion is broken down to show the relative amounts of various lipids. It is an interesting fact that the quinone—α-tocopherol portion contributes more to the lipid weight than the total carotenoids. The known components make up roughly 80% of the total lipids.

The unidentified portion (about 20%) probably consists mostly of minor constituents that have been reported to occur in chloroplasts. Either they had not been determined quantitatively, or else the values available are inaccurate. Among these substances are free fatty acids, free phytol, protochlorophyll, paeophytin, xanthophyllesters, antheroxanthin, phytoene, phytofluene, tocopherols, and tocopheryl quinones other than α-T and α-TQ, as well as other not yet identified lipids.

The lipid composition shown here is undoubtedly subject to considerable variation from species to species, as well as within a single plant species under differing physiological conditions. However, such a catalog is useful, for it provides in one place enough information on which to construct an accurate model for the quantum-conversion apparatus in photosynthesis.

Representative distribution of substances in chloroplast lamellae on basis of minimum molecular weight per atom of manganese of 960,000.

Lipid				
115 chlorophylls[1]			103,200	
	80 chl. a	71,500		
	35 chl. b	31,700		
24 carotenoids[2]			13,700	
	7 β-carotene	3,800		
	11 lutein	6,300		
	3 violaxanthin	1,800		
	3 neoxanthin	1,800		
23 quinone comp.[2]			15,900	
	8 plastoquinone A	6,000		
	4 plastoquinone B	4,500		
	2 plastoquinone C[11]	1,500		
	4.5 α-tocopherol	1,900		
	2 α-tocopherylquinone	1,000		
	2 vitamin K_1	1,000		
57 phospholipids[8] (phosphatidylglycerols)			43,000	
75 digalactosylglyceride[8]			69,500	
172 mongalactosyldiglyceride[8]			132,300	
23 sulfolipid[8]			19,100	
sterols[4]			ca 8,300	
unidentified lipids			90,000	
				495,000
Protein				
4,690 N atoms as protein			464,000	
1 Mn			55	
6 Fe			336	
3 Cu			159	
				465,000
			Lipid + Protein	960,000

5. METAL CHELATES AND PHOTOCHEMISTRY OF FLAVINS

G. K. Radda

I. Summary

In contrast to results of previous investigations it is shown by spectroscopic, ESR, and preparative methods that riboflavin or its semiquinone radical does not form stable manganese complexes. An explanation of this structurally unexpected phenomenon is advanced in terms of preferential enolization of the 2-carbonyl rather than that of the 4-carbonyl group of the isoalloxazine ring. The possible factors responsible for the formation of the unusually stable silver complex of riboflavin are also discussed.

The photoreduction of riboflavin, FMN, 6, 7-dimethyl-9-hydroxyethylisoalloxazine, and lumiflavin, in the absence of added electron donors, is studied. It is shown that lumiflavin, unlike the other three compounds, does not undergo photobleaching. It is concluded that the hydrogens for the reduction are derived from the side chain in an intramolecular rearrangement. Evidence for this is given by a detailed kinetic analysis of the reduction with and without external reducing agents.

The effects of metal ions (Ag^{+}, Cu^{++}, Ni^{++}, Mn^{++}, Na^{+}) and of the pH on the rate of reduction of FMN are measured, and a working hypothesis for the mechanism of the reaction is presented.

II. Metal Complexes

A. Introduction

The similarity in structure of 8-hydroxyquinoline and of the enol form of riboflavin led Albert to investigate the complexing properties of riboflavin with transition metals.[1] The stability constants derived in the early titration measurements were recently challenged by Harkins and Freiser.[2] These authors, however, still obtained measurable stability constants for complexes of certain metals, e.g., Cu^{++}. Hemmerick and Fallab, on the other hand, found no evidence for the formation of the cupric complex.[3] Foye and Lange[4] prepared a number of solid "chelates" between metals such as Cu^{++}, Fe^{++}, Mn^{++}, etc., and riboflavin which contained two metal ions per flavin.[4] The visible spectra of these chelates were found to differ "slightly but significantly from riboflavin and metal taken separately."[5]

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1. A. Albert, Biochem. J. 54, 646 (1953).
2. T. R. Harkins and H. Freiser, J. Phys. Chem. 63, 309 (1959).
3. P. Hemmerich and S. Fallab, Helv. Chim. Acta 41, 498 (1958).
4. W. O. Foye and W. E. Lange, J. Am. Chem. Soc. 76, 2199 (1954).
5. H. R. Mahler, A. S. Fairhurst, and B. Mackler, J. Am. Chem. Soc. 77, 1514 (1955).

The potential importance of such complexes in biological systems[6] prompted this investigation. Manganese was chosen for the main part of the study because of its presence in the chloroplast[7] and its ability to undergo riboflavin-sensitized photooxidation,[8] and because the visible spectra of the manganese complex was reported to exhibit the largest difference from that of the free flavin.[5]

B. Results

The possibility of complex formation between manganese and riboflavin was investigated by three methods.

1. Spectrophotometry The visible and ultraviolet spectra of 1:1 molar mixtures of riboflavin and $MnCl_2$ (conc. 0.5×10^{-4} M) were recorded at a series of pH values (from pH = 4 to 9) and compared with those obtained with riboflavin alone both by difference spectroscopy and by direct measurements. No significant differences were observed at pH values below 8, but a very small change was discernible at pH 9. This, however, could be attributed to light scattering as a result of the appearance of colloidal manganeous hydroxide in the solution. When two-, five-, and tenfold excesses of the manganous salt were used, no evidence for complex formation was found.

The sensitivity of the spectroscopic method for detecting small differences could in theory be increased by recording difference spectra at higher concentrations of both components. For reasons of solubility, FMN (flavin mononucleotide) was used instead of riboflavin in the following study.

The possibility of dimerization of the highly unsaturated flavin cannot be ruled out at high concentrations. In order to define this limitation of the spectroscopic method for investigating complexing, the absorption spectrum of FMN as a function of concentration was studied by using cells of thickness from 1 cm to 0.005 cm. The results are recorded in Fig. 5-1. No further changes could be observed on tenfold dilution of the solution at 0.5×10^{-4} M concentration. Since spectroscopically observable dimerization occurs at concentrations higher than 10^{-4} M, changes in the absorption spectrum on addition of metals could result from changes in the aggregation state of the dye molecules. That such changes do in fact occur is shown in Fig. 5-2. These curves were obtained by recording the spectral differences between a solution of FMN (10^{-2} M in 1-cm cell) and that of FMN with added metal salts. Both Ni^{++} and Mg^{++} ions cause an increase in dimerization, as observable from the increased absorption at the high end of the FMN spectrum (510 to 520 mμ). Since Mg^{++} is known not to form complexes with riboflavin, the spectral changes must be attributed to changes in the dielectric constant of the medium. A similar change is produced by Na^{+} ions again added as the chloride), but to a lesser extent even though they were added at such concentrations as to result in the same change in the dielectric constant of the

6. H. R. Mahler in Advances in Enzymology, Ed. by F. F. Nord (Interscience Publishers, Inc., New York, 1956), Vol. 17, p. 233.
7. J. V. Possingham and D. Spencer, Australian J. Biol. Sci. 15, 58 (1962).
8. A. A. Andreae, Arch. Biochem. Biophys. 55, 584 (1955).

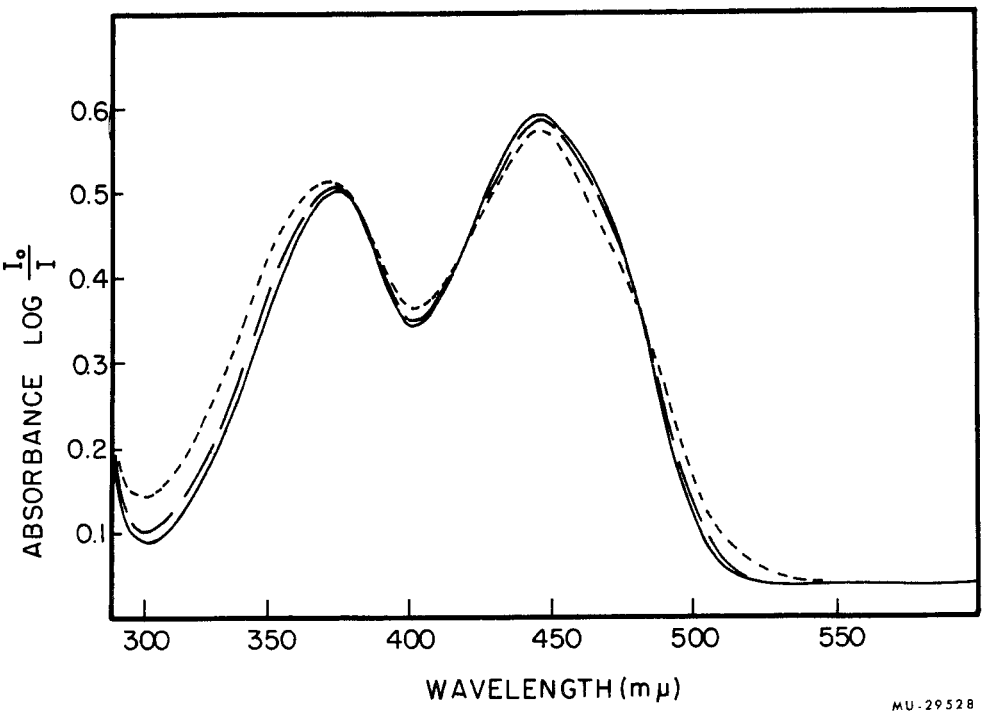


Fig. 5-1. Concentration dependence of the visible and ultraviolet spectra of FMN.
———— 0.5×10^{-4} M FMN in 1-cm cell;
— — — 1.0×10^{-3} M FMN in 0.05-cm cell;
-------- 1.0×10^{-2} M FMN in 0.005-cm cell.

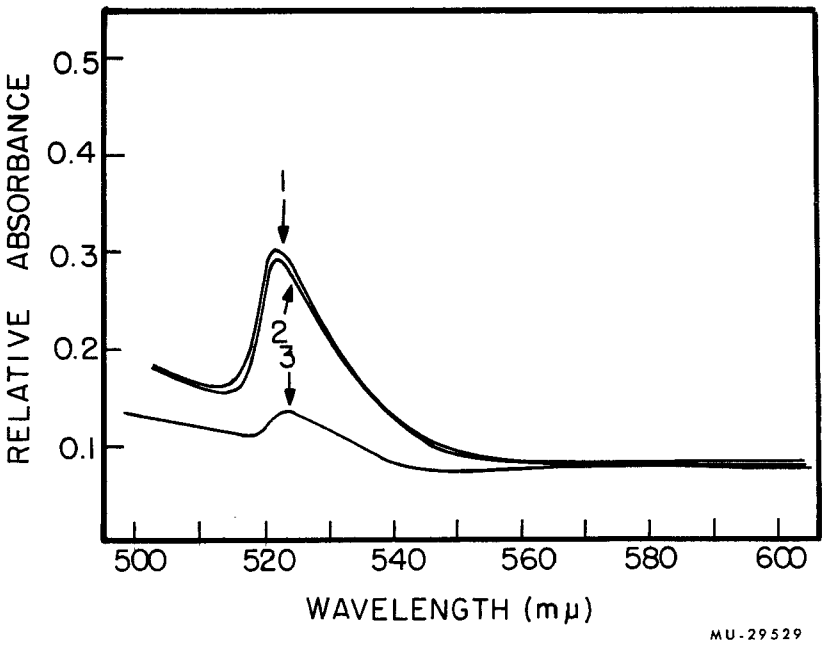


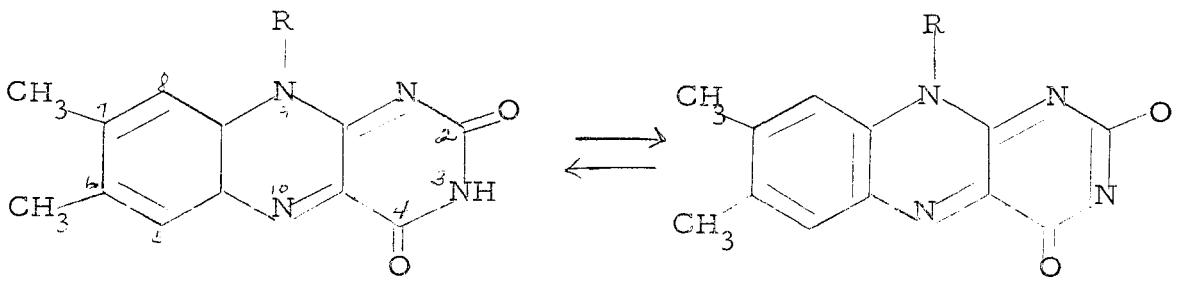
Fig. 5-2. Difference spectra of solutions of FMN with and without metal ions.
Curve 1: 10^{-2} M FMN + 10^{-2} M $MgCl_2$ vs 10^{-2} M FMN;
Curve 2: 10^{-2} M FMN + 10^{-2} M $NiCl_2$ vs 10^{-2} M FMN;
Curve 3: 10^{-2} M FMN + 3×10^{-2} M NaCl vs 10^{-2} M FMN.

solution as was caused by divalent metal salts. Evidently the effect of the metal ions on the state of aggregation of FMN is not a direct function of the dielectric constant of the medium but depends also on the charge (and presumably size) of the metal ions. It is therefore not possible to investigate complex formation at these increased dye concentrations.

2. Electron spin resonance The ESR spectrum of divalent manganese consists of six equivalent lines with an overall width of 770 gauss. Complexing agents affect this spectrum in various degrees, resulting in a broadening of the individual hyperfine lines or sometimes even a complete disappearance of the signal. When FMN (10^{-2} M) is added to a solution of manganous chloride (10^{-3} M) at pH 6, no change in the ESR spectrum of the manganous ions can be observed.

3. Isolation of the "chelate" The alleged riboflavin manganese complex was prepared by the method of Foye and Lange.[4] This requires the precipitation of the complex from a solution of riboflavin at pH 9 by adding to it a solution of $MnCl_2$ simultaneously with 0.1 N NaOH so as to maintain the pH of 9. The precipitate thus obtained contained manganese (shown by qualitative tests using persulfate oxidation to permanganate). Quantitative analysis after purification[4] gave the following composition: C, 49.64%; H, 5.59%; N, 13.59%; MnO_2, 5.9%. This composition does not correspond to any stoichiometrically feasible complex. It approximates most closely the formula Rf_2Mn, in contrast to $Rf \cdot Mn_2 \cdot 2H_2O$ reported by Foye and Lange.[4] It is essential to note that these authors based their formula entirely on the values of microanalysis for carbon, hydrogen, and water, the last of which is in serious error when compared with the theoretical value. Since the reported composition of the complex may have been in error, the significant difference between the extinction coefficient of riboflavin and the complex observed by Mahler et al.[5] on the complex prepared by Foye and Lange is not meaningful, although the observed increase in absorption at 500 mμ remains to be accounted for. Indeed, the complex prepared in this work also shows a slightly increased absorption at 500 mμ, both in aqueous solution and in dimethylsulfoxide. If, however, the solutions were left standing for 48 hr, the original riboflavin spectrum was recovered. Similarly, the infrared spectrum of the "complex" (taken in a Nujol suspension) is different from that of riboflavin in the carbonyl region (Fig. 5-3).

To investigate both these spectral observations, a solution of riboflavin was treated in the same way as in the preparation of the manganese complex, except that the reprecipitation was brought about by acidifying the solution instead of adding a solution of manganese. The spectral characteristics (both visible and infrared) of the precipitate thus obtained were identical with those of the "manganese complex." It is possible that these spectroscopic differences represent an enolization in the isoalloxazine ring of the flavin:



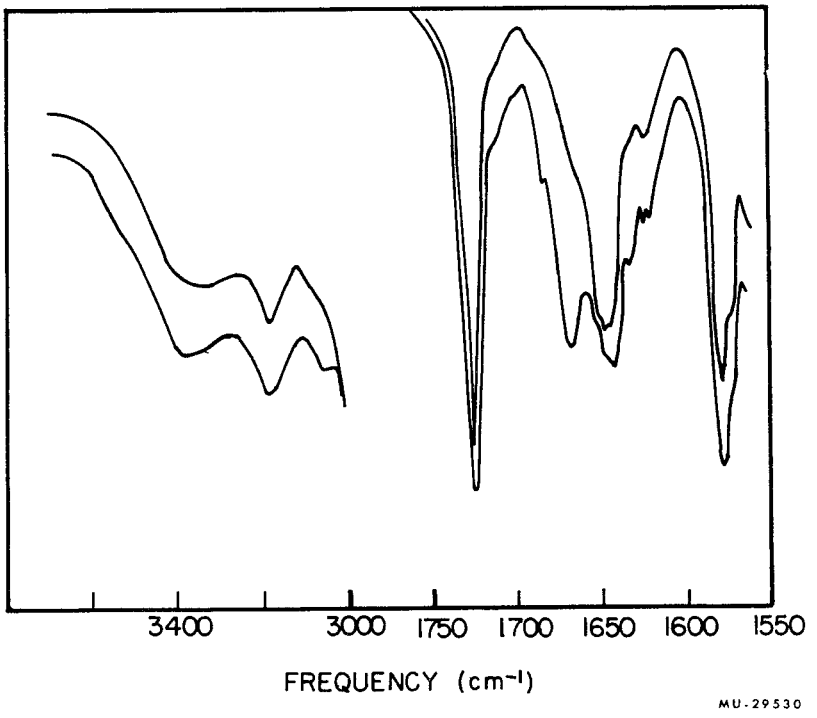


Fig. 5-3. Infrared spectra of
(lower curve) riboflavin (plates, mp 275-280°), and
(upper curve) riboflavin manganese complex.

It is likely that in the reprecipitation procedure imperfect crystals are formed. It is therefore of interest to determine whether the two known crystalline modifications of riboflavin[9] may be a result of this type enolization. Some needlelike crystals were obtained on recrystallization from dilute (1%) acetic acid (mp 292°C), and platelike crystals from water (mp 275-280°C). The infrared spectrum of the former is shown in Fig. 5-4 (in the critical regions) and that of the latter form in Fig. 5-3. The disappearance of the band at 1670 cm^{-1} and the appearance of a sharp band at 3500 cm^{-1} on going from the low-melting form to the high-melting one may represent the change of a keto group to an enolic hydroxyl. Similarly, the lack of the weak band at 3100 cm^{-1} in the high-melting form (Fig. 5-4) may be due to the disappearance of the N-H stretching. All the bands at the region 3000 to 3600 cm^{-1} represent exchangeable hydrogens (Fig. 5-4). This interpretation is subject to the limitations imposed by the necessity of observing the spectra in the solid state. If, however, these changes are a result of enolization, then it is clear from studies on analogous models that it is the carbonyl group at the 2 position that is involved in the process.[10] This conclusion is consistent with the observation that on treatment with strong alkali riboflavin hydrolyzes to urea and quinoxalinecarboxylic acid,[11] and with the failure to obtain derivatives of lumiflavin with substituents $-OCH_3$ and $-SCH_3$ at the 4 position[12] whereas stable derivatives can be obtained when these substituents occupy the 2 position.[13]

a. In conclusion, the riboflavin manganese "complex" obtained by the precipitation method of Foye and Lange[4] is probably a result of coprecipitation of riboflavin with manganous hydroxide, while the spectral differences observed by Mahler et al. are due to enolization. Furthermore, the apparent instability of the chelates of riboflavin with transition metals can be accounted for in terms of preferential enolization of the 2-carbonyl group rather than that of the 4-carbonyl.

b. Riboflavin silver complex. The stoichiometry and visible spectrum of the stable riboflavin silver complex has been studied by several workers.[14,15] It has been suggested that the structure of the 1:1 complex is of the dipolar type:

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9. S. Shimizu, Vitamins (Kyoto) 10, 24 (1956); Chem. Abstr. 51, 15638a (1957).
10. Randall, Fowler, Fuson, and Dangl, Infrared Determination of Organic Structures (D. van Nostrand Co., Inc., Princeton, 1949).
11. H. H. Fall and H. G. Petering, J. Am. Chem. Soc. 78, 377 (1956).
12. P. Hemmerich, B. Prijs, and H. Erlenmeyer, Helv. Chim. Acta 42, 2164 (1959); P. Bamberg, P. Hemmerich, and H. Erlenmeyer, Helv. Chim. Acta 49, 395 (1960).
13. F. Müller, P. Hemmerich, and H. Erlenmeyer, Experientia 18, 497 (1962).
14. P. Bambert and P. Hemmerich, Helv. Chim. Acta 44, 1001 (1961).
15. I. F. Baarda and D. E. Metzler, Biochim. Biophys. Acta 50, 463 (1961).

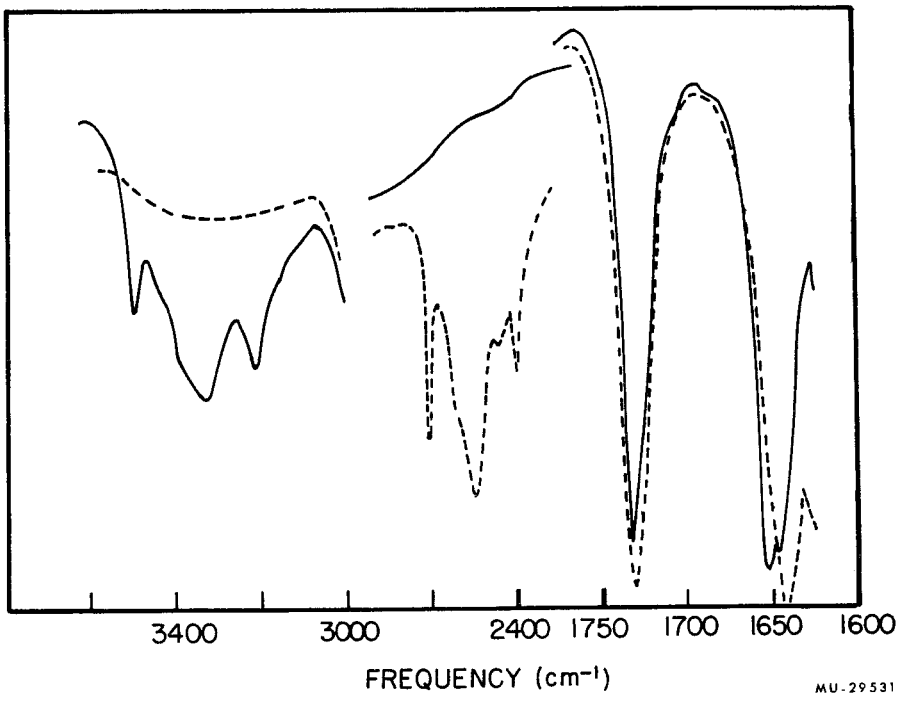
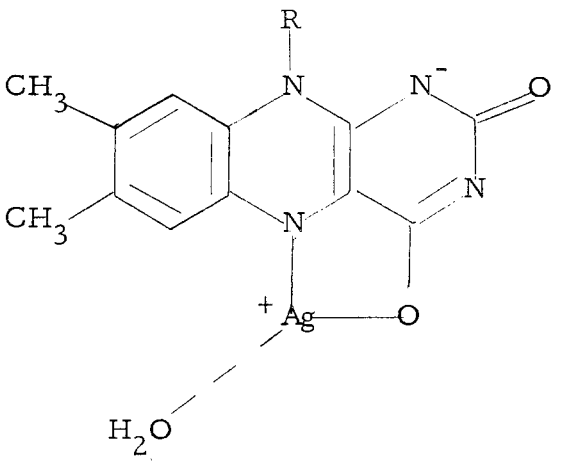


Fig. 5-4. Infrared spectrum of riboflavin.
——— needles, mp 292°
------- partially deuterated.



We have now obtained some evidence for this structure from the infrared spectrum of the complex (prepared as a solid, taken in Nujol suspension). The salient feature of the spectrum is the disappearance of the band at 1730 cm^{-1} observed in riboflavin and assigned to the 4-carbonyl group. This suggests that Ag^{+} ions may change the mechanism of enolization of riboflavin prior to the formation of the complex. (The complex is formed even at low pH's.) If silver ions donate an electron (probably only partially) to the isoalloxazine ring (e.g., to form a charge-transfer complex) the flavin nucleus will be similar to the semiquinone. Now, it has been shown that when a substituent $-X$ in the 4 position is single-bonded to the flavin the resulting compound is unstable, but that the corresponding free radical (with an additional electron) is stable.[16] It is in this sense that the transfer of an electron from Ag^{+} to the nucleus contributes the necessary driving force for the enolization of the 4-carbonyl (hence the formation of a single bond between the 4-substituent and the ring) to be favorable.

c. Metal complexes of flavin semiquinone. As an outcome of the arguments presented above in some preliminary experiments, the effect of Mn^{++} ions on the flavin semiquinone was investigated by two methods:

(i) The visible spectrum of a half-reduced solution of riboflavin contains a small peak at 570 mμ which has been assigned to the semiquinone radical.[17] When Mn^{++} ions are added to a degassed solution of half-reduced FMN, no change in the spectra can be observed (either by difference or by direct measurements) even on an expanded scale.

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16. P. Bamberg, P. Hemmerich, and H. Erlenmeyer, Helv. Chim. Acta 49, 395 (1960).

17. Q. H. Gibson, V. Massey, and N. M. Atherton, Biochem. J. 85, 369 (1962).

(ii) The ESR spectrum of the radical has been reported by several authors; it shows at least 14 hyperfine lines,[17, 18] and as many as 32 under better conditions.[19]

We were able to observe 14 hyperfine lines, both when the radical was generated by half reducing a solution of FMN (10^{-2} M) with sodium dithionite,[17] and when it was formed by irradiating a degassed solution of FMN (10^{-3} M) by visible light. When the latter solution contained Mn^{++} ions (10^{-4} M) at pH 3, only 12 lines were observed. The two weakest lines could not be detected although the other lines were as distinct as or better than when no Mn^{++} ions were present (Fig. 5-5). Since this effect could be due to a number of causes, interpretation must await further experimentation.

III. Photochemistry

Riboflavin (I, a) has long been known to be decomposed to lumiflavin (I, b) and lumichrone (I, c) as well as fragments of the ribityl side chain under illumination with visible light in the presence of oxygen.[20] Under anaerobic conditions irradiation brings about spectroscopic changes which are the same as those produced by reducing agents such as sodium dithionite or Zn-HCl. It was suggested by Strauss and Nickerson that the photolysis resulted in the splitting of water to provide the hydrogen for the reduction of the flavin and hydroxyl radicals which underwent secondary reactions.[21] Evidence for this mechanism was presented by several workers.[22] An alternative hypothesis that the side chain of riboflavin is involved in the photoreduction[23] has not been well substantiated, although on energetic grounds it appears to be a more feasible explanation.

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17. Q. H. Gibson, V. Massey, and N. M. Atherton, Biochem. J. 85, 369 (1962).
18. B. Commoner and B. B. Lippincott, Proc. Natl. Acad. Sci. U. S. 44, 1110 (1958).
19. A. Ehrenberg, Arkiv Kemi 19, 97 (1962).
20. R. Kuhn and Th. Wagner-Jauregg, Ber. Deut. Chem. Ges. 66, 1577, 1950 (1933).
21. G. Strauss and W. J. Nickerson, J. Am. Chem. Soc. 83, 3187 (1961).
22. W. J. Rutter, Acta Chem. Scand. 12, 438 (1958); L. P. Vernon, Biochim. Biophys. Acta 36, 177 (1959).
23. B. Holmström and G. Oster, J. Am. Chem. Soc. 83, 1867 (1961).

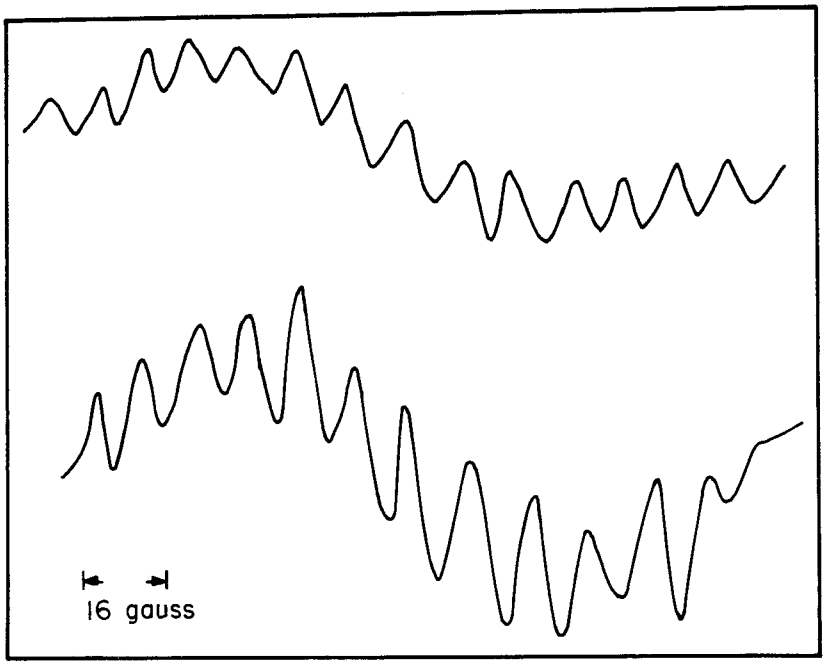
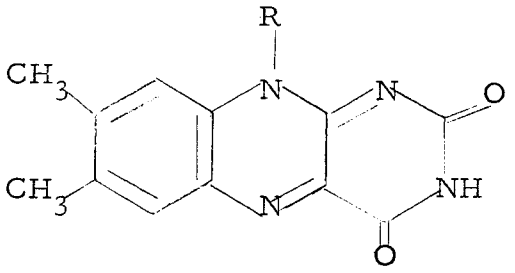


Fig. 5-5. ESR spectra of FMNH·,
Upper curve: no metal ions.
Lower curve: in presence of 10^{-4} M Mn^{+} ions.

A. Results and Discussion

The photoreduction of solutions of riboflavin (I, a), lumiflavin (I, b), and 6, 7-dimethyl-9-hydroxyethylisoalloxazine (I, d) in phosphate buffer at pH 7 was investigated spectrophotometrically.



I, a R = $-CH_2-CHOH-CHOH-CHOH-CH_2OH$

I, b R = $-CH_3$

I, c R = $-H$

I, d R = $-CH_2-CH_2OH$

The solutions were thoroughly deoxygenated by bubbling argon through them in a closed system[24] for 1 hour, and the spectra were recorded immediately after illumination with a 500-W tungsten lamp. The diminution in the 445-mμ absorption band as a function of illumination time for each compound is recorded in Fig. 5-6. No change can be observed for lumiflavin, but riboflavin and compound (I, d) were photobleached. When air was admitted, the original spectra were only nearly restored. On addition of tenfold excess of ribose or twentyfold excess of ethanol to the solution of lumiflavin, a small decrease of the 445-mμ absorption was observed on illumination (see Fig. 5-6), and the original spectra were completely restored by oxidation.

These results clearly indicate that at least a hydroxyethyl side chain is necessary for the photoreduction, and that water cannot be responsible for the reaction because then lumiflavin, which is electronically identical to riboflavin and compound (I, d), should undergo the same reaction. To show, however, that this is not a rate phenomenon perhaps associated with a property of the molecules not manifest in the absorption spectra,[25] the rates of photoreduction of riboflavin, FMN, FAD, and lumiflavin upon use of an external reducing agent (EDTA) were compared. In these experiments as in those of Fig. 5-6, a constant light intensity was obtained by setting the powerstat to the same value. (It was shown later that this way the intensity does not vary by more than 5%.) In addition, here an interference filter was used which cuts off the light at wavelengths below 400 mμ. The rates of reduction of each compound except that of FAD are identical within the limits of experimental error (Fig. 5-7). This is more forcibly represented in Fig. 5-8. These graphs were derived by assuming the following mechanism for the photoreduction:

24. P. A. Loach, in Bio-Organic Chemistry Quarterly Report, UCRL-10032, Jan. 1962, p. 45.

25. It was recently suggested [L. R. Tether and J. H. Turnbull, Biochem. J. 85, 517 (1962)] that the side chain of riboflavin promotes the transition from the first excited singlet to the triplet, and that this effect is more pronounced in FMN and less so in lumiflavin.

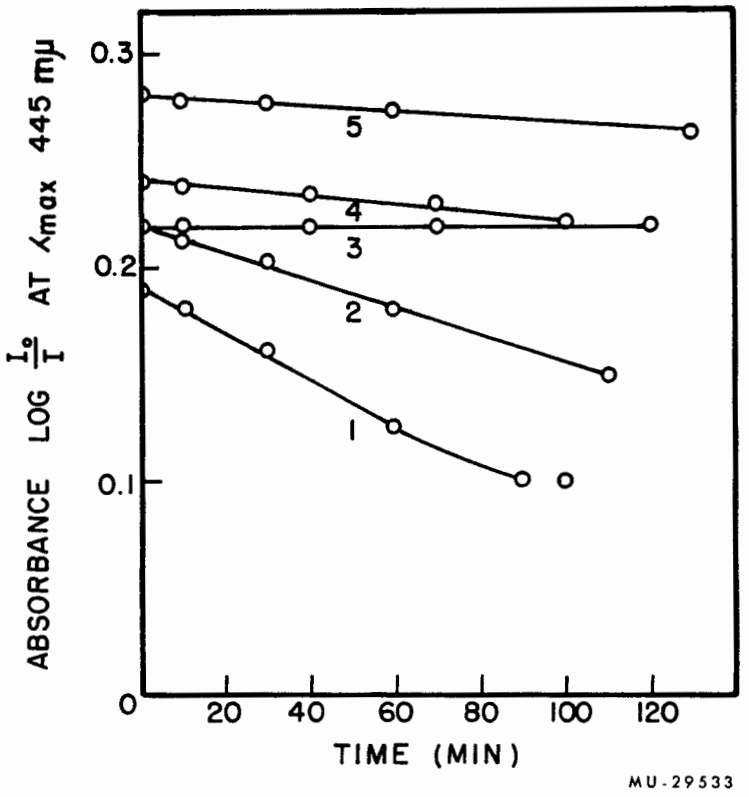


Fig. 5-6. Photoreduction of flavins (pH 7; phosphate buffer).
Curve 1: riboflavin.
Curve 2: 6,7-dimethyl-9-hydroxyethylisoalloxazine.
Curve 3: lumiflavin.
Curve 4: lumiflavin + ribose (tenfold excess).
Curve 5: lumiflavin + ethanol (fortyfold excess).

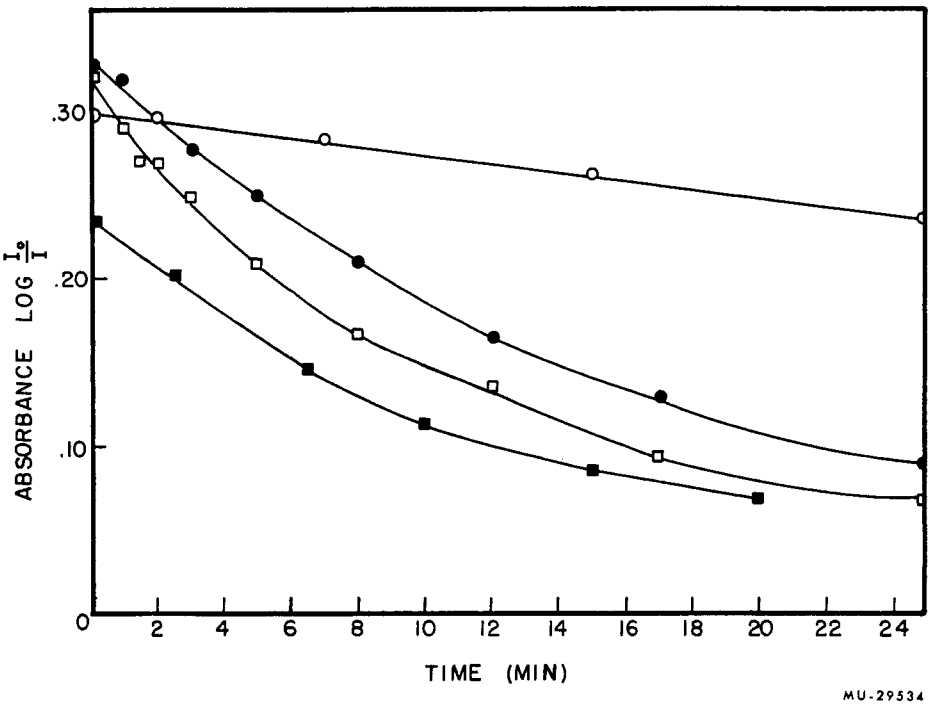


Fig. 5-7. Rate of photoreduction of flavins with EDTA (fortyfold excess), pH 7, phosphate buffer.

○ : FAD; □ : riboflavin;
● : FMN; ■ : lumiflavin.

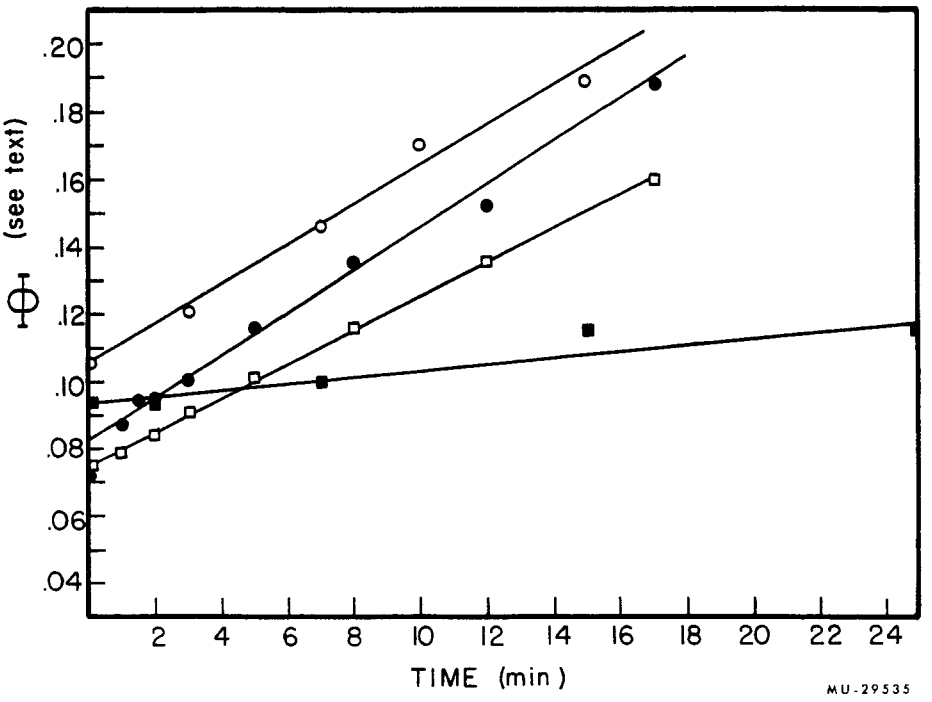


Fig. 5-8. Steady-state plot for photoreduction of flavins.
○: lumiflavin; □: FMN;
●: riboflavin; ■: FAD.

$$R + h\nu \xrightarrow{k_1} R^*, \quad \text{where R = flavin,} \tag{1}$$

$$R^* + S \xrightarrow{k_2} R, \quad \text{where S = solvent,} \tag{2}$$

$$R^* + B \xrightarrow{k_3} \text{Products, where B = EDTA.} \tag{3}$$

Equation (1) represents the light-induced excitation of the flavin to a reactive state (either to the first singlet or indirectly to the triplet), Eq. (2) the solvent quenching of the reactive state, and Eq. (3) the reduction step. Since S and B are in large excess (EDTA was used in a fortyfold excess) their concentration terms in the rate expressions can be included in constants. The rate of quanta absorbed = $k_1 I_{abs} = I_o - I$; $I = I_o e^{-[R]\cdot\epsilon}$. Using a steady-state treatment, one can obtain rate expression

$$\frac{-d[R]}{dt} = \frac{k_1 \cdot k_3 \cdot I_o (1 - e^{-\epsilon e[R]})}{k_2[S] + k_3[B]} ;$$

and, on integration,

$$-\underbrace{\left[[R] + \frac{1}{\epsilon e} \log (1 - e^{-\epsilon[R]}) \right]}_{\phi}^{R_2}_{R_1} = \frac{k_1 \cdot k_3 \cdot I_o}{k_2[S] + k_3[B]} (t_2 - t_1)$$

Knowing the extinction coefficients, one can calculate the concentrations. The plots for ϕ vs time for the four reactions are shown in Fig. 5-8. The slopes of the lines (the constants on the right-hand side of the equation) are equal for each compound save FAD. Since one can assume that the solvent quenching and the rate of quanta absorbed are identical for all three compounds with the parallel plots (their spectra are identical), the rates of reduction must also be equal. The reduction process is almost certainly in competition with solvent quenching, since the overall rate is dependent on the concentration of EDTA and on the light intensity, too. The rate expressions above are, of course, oversimplified, since terms for self-quenching ought to be included. Such quenching is probably more pronounced in the dinucleotide (perhaps through the formation of an intramolecular charge-transfer complex between the adenosine and flavin parts), which may account for its slower rate of reduction. The linearity of the plots, to 60 to 70% of the reaction, however, is consistent with a mechanism of the type represented by Eqs. (1) through (3).

In all the cases in which an "external" reducing agent is employed for photoreduction, reoxidation restores the original spectra--in contrast to the bleaching of riboflavin without an external electron donor. This again supports the idea that in the latter case degradation of the compound takes place prior to the oxidation step.

Further details about the mechanism of the reaction were sought as follows.

1. Rate of reduction of lumiflavin

Figure 5-6 shows that the rate of reduction of lumiflavin with excess of ribose or ethanol is still slower than that of riboflavin or the 9-hydroxyethyl compound (I, d). These observations suggest that the reaction may follow an intramolecular path. To obtain more precisely defined data on this point, the rate of reduction of riboflavin was compared with that of lumiflavin with added ribitol. During these experiments the light intensity was constantly monitored by a Weston light meter and adjusted manually to the desired value if changes of more than 2% occurred. Under these conditions at the same initial concentrations of riboflavin and lumiflavin (pH 7, phosphate buffer) the half-life of reduction for riboflavin was 90 minutes, whereas a 1:1 mixture of lumiflavin and ribitol showed no change during this time. In fact, it was necessary to increase the concentration of ribitol to eightyfold excess and the light intensity by a factor of two (from 2000 to 4000 foot candles) before the reduction of lumiflavin by ribitol approached the same rate as that obtained for riboflavin. (Lumiflavin is again not reduced under these conditions without added ribitol.) This clearly indicates that the rate of intermolecular reduction by ribitol is negligible compared with that of the intramolecular process.

2. Effect of metal ions

The effect of added metal ions on the rate of photoreduction of FMN in an unbuffered solution was studied (the pH was adjusted to 5.3 when necessary). The results are shown in Fig. 5-9. Mn^{++} and Ni^{++} ions decreased the initial rates to 1/3 and 1/5, respectively, whereas Na^{+} ions had no effect on the reaction. (These salts were added as chlorides.) These data suggest that the paramagnetism of the two transition metals is responsible for the decreased rates and hence the activated state may involve a triplet. The complete inhibition of the reaction by Ag^{+} ions is probably due to its ability to form strong complexes with riboflavin that are nonfluorescent (and presumably nonphosphorescent). The increased absorption obtained on irradiation of solutions of riboflavin in presence of Cu^{++} ions is a result of the photoreduction of Cu^{++} ions, since Cu^{+} ions, like silver, also form stable complexes with riboflavin. This is supported by the observation that when ascorbic acid is added to a mixture of riboflavin and cupric ions in the dark, spectroscopic changes take place that are similar to those in the photoreduction described above.

3. Effect of pH

The rate of photoreduction as a function of pH is shown in Fig. 5-10. Increasing concentration of hydrogen ions decreases the rate of reduction. This is inconsistent with the general acid-base catalysis of the reaction shown by Holmstrom and Oster,[23] but is consistent with Halwer's observations on the aerobic photobleaching of riboflavin.[26] He has found that this, too, is subject to general acid-base catalysis, but that hydrogen ions have a specific

26. M. Halwer, J. Am. Chem. Soc. 73, 4870 (1951).

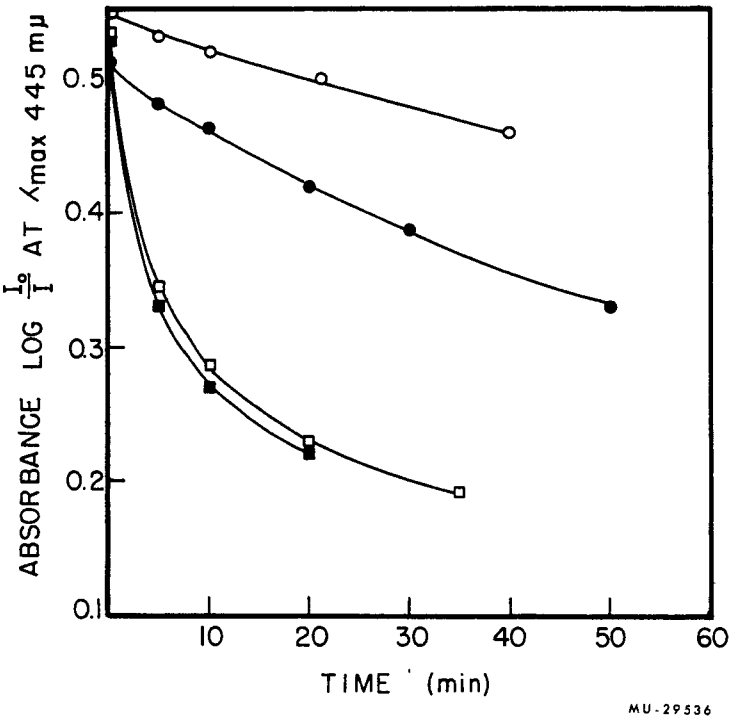


Fig. 5-9. Effect of metals on the rate of photoreduction of FMN (0.5×10^{-4} M).
○: FMN + 1×10^{-3} M $NiCl_2$, pH 5.3;
●: FMN + 1×10^{-3} M $MnCl_2$, pH 5.3;
□: FMN + 2×10^{-3} M NaCl, pH 5.3;
■: FMN at pH 5.3.

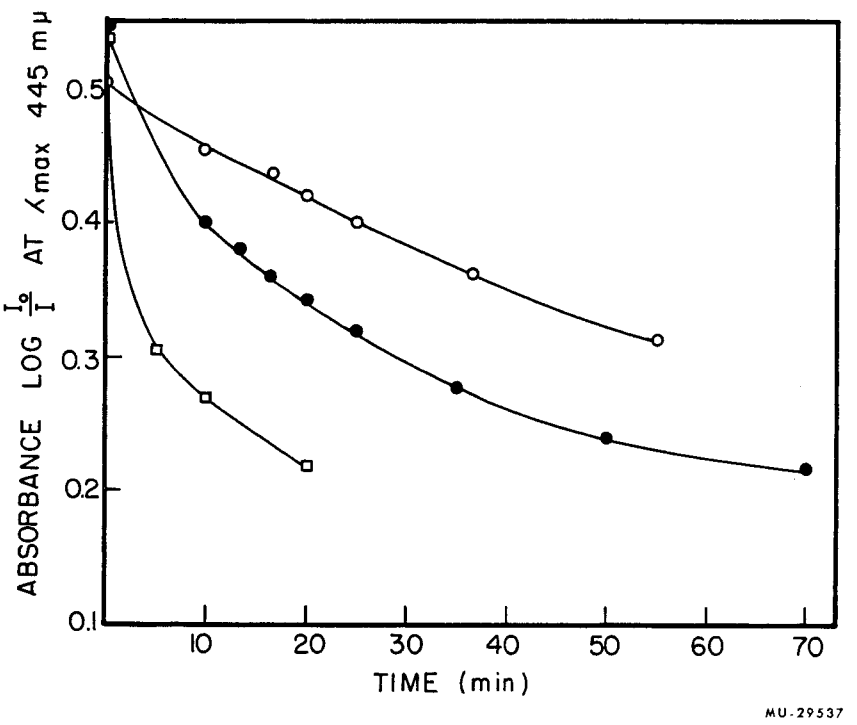


Fig. 5-10. The pH dependence of the rate of FMN photoreduction
○: pH 3; ●: pH 5.0; □: pH 5.3.

negative catalytic effect on the rate. We have observed the same kind of effect on pH on the rate of appearance of the semiquinone radical as observed by electron spin resonance (Fig. 5-11), but as yet it is not known whether the radical is a true intermediate of the reaction or is produced in an equilibrium between the completely reduced and oxidized forms of FMN after the photoreduction. It is known, for instance, that when FMN is reduced by sodium borohydride (truly not a one-electron reducer) the semiquinone can still be observed.

B. Conclusions

It is possible to suggest a mechanism as a working hypothesis as follows:

1. Flavin is excited by light to the first singlet, which then undergoes a radiationless slow transition to the triplet.

2. A hydrogen atom from No. 2' carbon of the side chain (or possibly from the hydroxyl group on this carbon) is transferred to the nucleus in an intramolecular shift, producing the semiquinone. (It is possible, however, that if the first triplet can be written as a highly polar state, a hydride ion is shifted.)

3. This is followed by the transfer of an electron from the aliphatic free radical on the side chain to the nucleus, the loss of a proton at the oxygen of C_2', and the gain of proton on the nucleus. (This last proton shift, which may not follow the sequence indicated, could be the step responsible for the observations presented in III. A. 3.)

Experiments to test this hypothesis are in progress.

The author wishes to thank Miss Ann Maksim for her help in the ESR work.

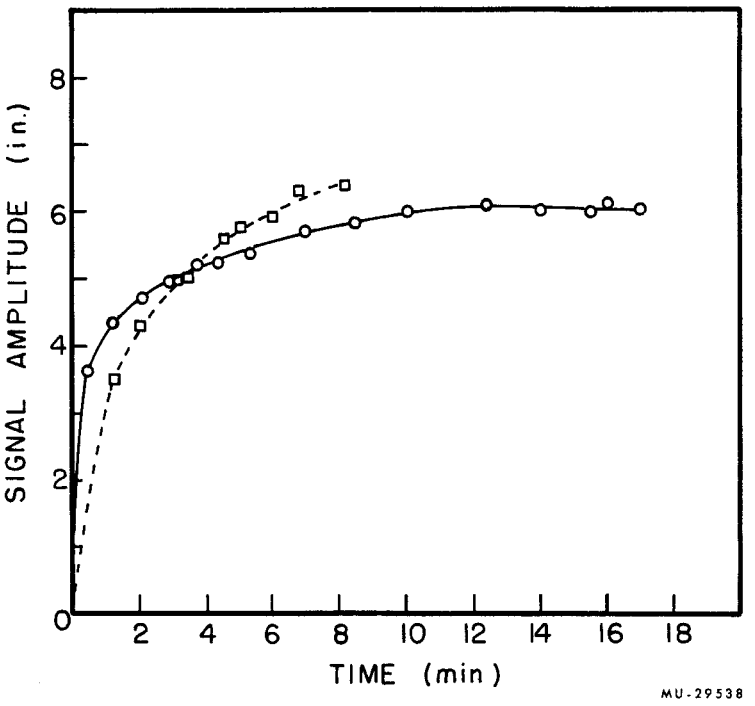


Fig. 5-11. Rates of appearance of FMNH· on illumination.
○ : FMN (10^{-3} M), pH 5.5; □ : FMN (10^{-3} M), pH 3.0.

6. PHOTOINDUCED ESR IN SOME SOLUTIONS OF ORGANIC ELECTRON DONORS AND ACCEPTORS

David F. Ilten and Maurits E. Kronenberg

In hopes of ultimately understanding the quantum-conversion process in photosynthesis, much work has been devoted to the study of model systems that simulate phenomena thought to occur in plants. Electron-transfer processes have been of particular interest in this Laboratory. Kearns studied electron-transfer phenomena in the solid state.[1] Eastman continued work on solids, but also used ESR to attempt to detect a rapidly reversible electronic transfer between donor and acceptor molecules in solution.[2] Such a solution would be expected to exhibit electronic conductivity if the electrons were free to migrate from molecule to molecule. The systems investigated, however, were generally quite reactive. No solution was found for which it could be claimed unequivocally that an electron was moving reversibly from a donor molecule to an acceptor molecule.

More recently Lagercrantz and Yhland have observed photoinduced ESR signals in solutions of p-chloranil in tetrahydrofuran and in several other systems.[3] They attributed these signals to the unpairing of the electrons involved in the formation of a charge-transfer complex between the donor (THF) and the acceptor (chloranil). The signals were approximately 15 gauss wide and exhibited rise times and decay times that were approximately 1 second, the fastest instrumental response time. If, in fact, these signals were caused by free, unpaired electrons, the solutions should show very interesting electrical and optical properties such as photoconductivity. It is the purpose of this work to attempt to duplicate some of the results of Lagercrantz and Yhland, to extend the studies to new systems, and to correlate these results with electrical conductance measurements.

Experimental Procedure

Much of the data presented in Table 6-I must be considered as exploratory in nature, because no rigorous purification of the sample materials was undertaken. However, the solvents were distilled and the solutes recrystallized. The ESR data were obtained by using a spectrometer consisting of a Varian magnet power supply, a Varian 100-kc phase-sensitive field modulation unit for crystal detection, and a magnet and 10-kc AFC unit constructed in this laboratory. The samples were tested for dark signals in quartz tubes of 4 mm o.d. and containing approximately 1/2 ml of solution in the cavity.

1. David R. Kearns, Electrical Properties of Organic Solids (thesis), UCRL-9120, March 1960.
2. John W. Eastman, Charge-Transfer Association and Paramagnetism in Some Organic Systems (thesis), UCRL-9722, Aug. 1961.
3. G. Lagercrantz and M. Yhland, Acta Chem. Scand. 16, 1043 (1962).

They were then illuminated in situ through the slots in the Varian No. 4531 rectangular cavity. The light source was an AH-6 high-pressure mercury lamp located 70 cm from the cavity. A Corning 0-52(7380) filter having about 20% transmission at 350 mμ, 50% at 360 mμ, and 90% at 400 mμ was used. The light was focused on the cavity slots with a Pyrex lens located approximately 15 cm from the cavity. In most cases, the samples were degassed.

Results

As indicated in Table 6-I, the systems investigated showed widely differing spin concentrations, rise times, and decay times. This certainly indicates a variety of processes. The rapid rise times and decay times may truly result from an electron-transfer process, although instrumentation with a more rapid response will be required to verify this. The system with slow rise times and decay times may involve an electron transfer as an initiating process, but this is undoubtedly followed by a "chemical" process, such as hydrogen abstraction.

An interesting system to discuss in some detail is p-chloranil dissolved in p-dioxane. The dioxane was twice distilled and then dried by refluxing over sodium and distilling. The p-chloranil was obtained from K&K Laboratories in Jamaica, New York, and then recrystallized from benzene as yellow needles approximately 2 cm long. A 0.05 M solution of p-chloranil in p-dioxane was prepared and degassed eight times in the sample tube by freezing, evacuating, and then melting. This sample showed no ESR signal in the dark.

Large signals were observed when the solutions were illuminated. The growth curve is shown in Fig. 6-1. It will be noted that the rise to maximum spin concentration was not instantaneous, but required approximately 1.5 minutes of illumination, probably far too long for a simple electron-transfer process. The decay was, likewise, a slow process, and is shown in Fig. 6-2. The decay curve can be analyzed kinetically as in Figs. 6-3 and 6-4. That the data do not fit second-order kinetics is shown by Fig. 6-3, where the reciprocal of the spin concentration is plotted against time. In Fig. 6-4 the decay curve is analyzed as a composite curve resulting from the presence of two paramagnetic species having half-lives of 0.75 min and 9 min, respectively. Figure 6-5 illustrates a peculiar light reaction occurring in a solution that has been illuminated for about 10 minutes. When the light is cut off by means of a shutter, there is an initial small increase in the signal level which is followed by a gradual decrease. Illuminating the sample again results in a rapid decrease, followed by a gradual increase in the signal level. Repeating the cycle shows an even more pronounced increase in spin concentration when the light is blocked off. These observations seem to indicate that at least two competing photoinduced processes are taking place, one producing spins, and the other resulting in the recombination or destruction of the species bearing unpaired electrons.

Table 6-I. Observations of photoinduced light signals in p-chloranil and other compounds in various solvents.

Solvents	Color of solution	Results (signals approx 15 gauss wide)
A. p-Chloranil solutions, 0.05 M		
Benzene	Yellow	No dark signal, no light signal
p-Xylene	Orange (yellow)	No dark signal, possible light signal
Mesitylene	Orange (yellow)	No dark signal, small light signal
Phenitol	Red (orange)	No signal
o-Chlorophenol	Orange (red)	No dark signal, small light signal, rapid rise and decay time ($< 1/10$ sec)
α-Chloronaphthalene	Red (orange)	No dark signal, no light signal
Pyridine	Dark solution (precipitate)	No dark signal, no light signal
Quinoline	Dark solution (some precipitate)	Very large dark signal, no fine structure. No appreciable enhancement in light (ca. 10^{18} spins/ml)
Tetrahydrofuran	Yellow	No dark signal, small light signal (approx 10^{15} spins/ml)
Thiophene	Orange	No signal
p-Dioxane	Yellow (green)	No dark signal, large light signal (approx 10^{17} spins/ml). Slow response enhancement when light turned off
Morpholine	Reddish-brown	Small dark signal, factor-of-10 increase in light, enhancement when light off.
Piperidine	Reddish-brown	No dark signal, small light signal, rapid response
1, 2-Dimethoxyethane	Yellow	No dark signal, small light signal, rapid response
Nujol (< 0.05 M)	Yellow solution	No dark signal, small light signal, long-lived
Triphenyl phosphate	Orange-red	No signal
B. Other systems		
o-Chloranil + thiophene (0.05 M)	Orange-red	No dark signal, small light signal about 30 gauss wide
o-Chloranil + diphenyl-ether (0.05 M)	Reddish	No dark signal, small light signal
TCNE[a] + diphenylether (0.05 M)	Reddish	No dark signal, small light signal
TCNE + pyridine	Dark brownish	Dark signal; 9-line fine structure
TCNE (0.05 M) pyridine (0.5 M) $EtCl_2$ Solvent	Dark brownish	Dark signal; 9-line structure
Perylene (0.001 M) + hexafluorobenzene	Yellow-green	No dark signal, small light signal

[a] TCNE = tetracyanoethylene

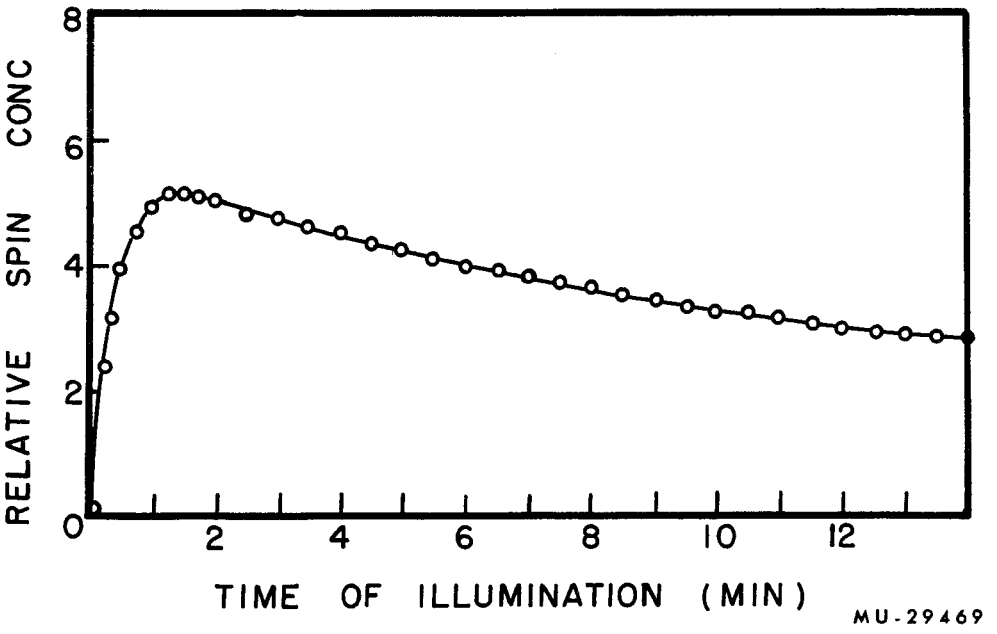


Fig. 6-1. Photoinduced ESR signals in 0.05 M solution of p-chloranil in dioxane.

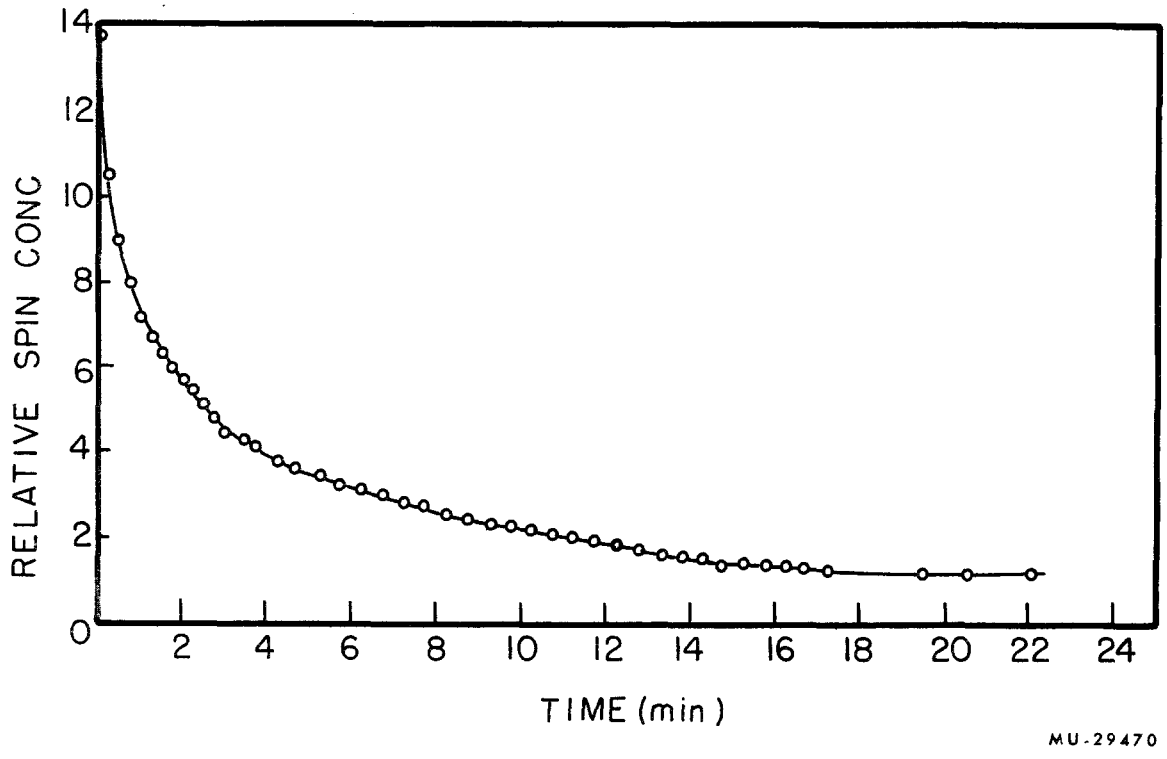


Fig. 6-2. Decay curve for photoinduced ESR signal in 0.05 M solution of p-chloranil in dioxane.

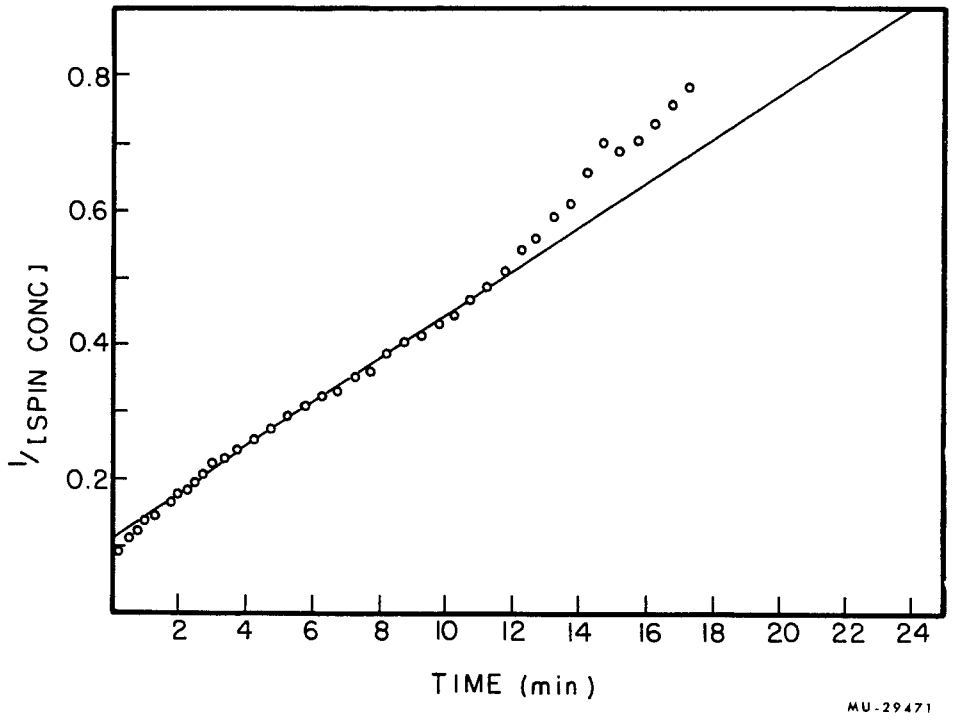


Fig. 6-3. Kinetic study of decay curve for photoinduced ESR signal in solution of p-chloranil in dioxane.

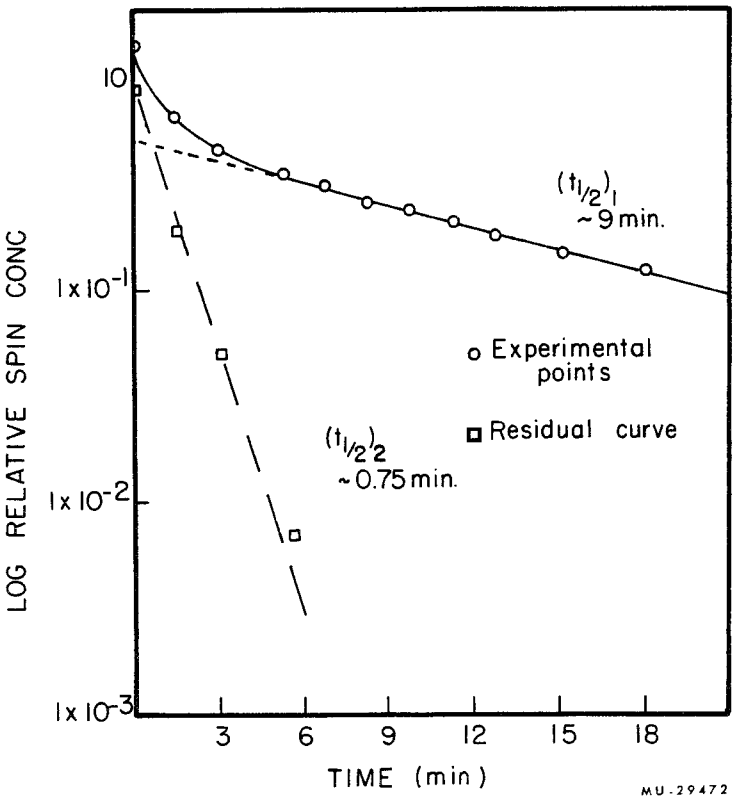


Fig. 6-4. Kinetic study of decay curve for photoinduced ESR signal in solution of p-chloranil in dioxane.

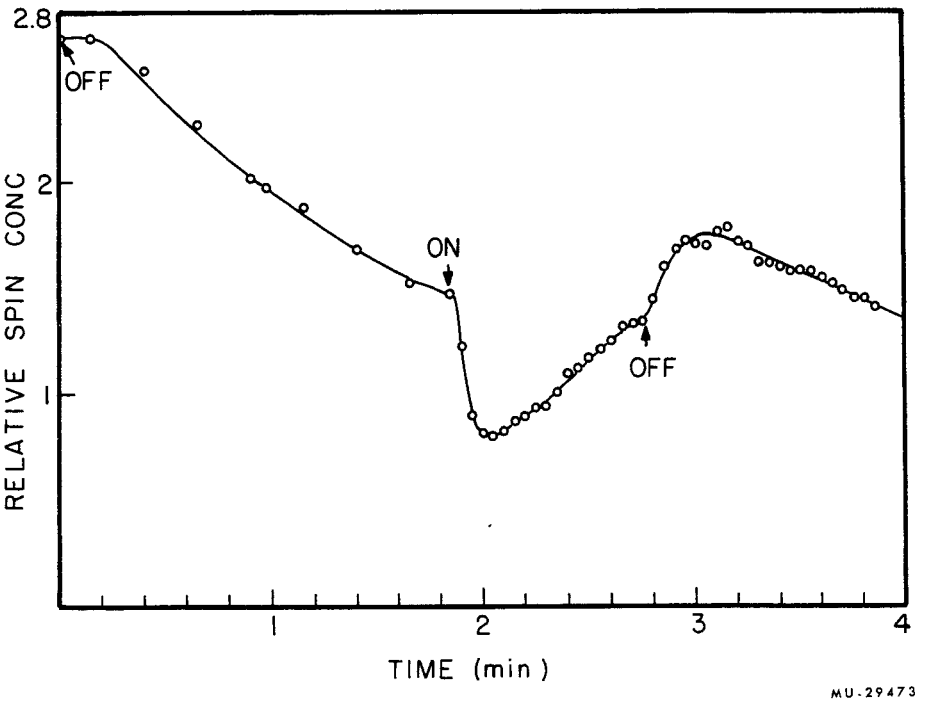


Fig. 6-5. Effects of illumination on spin cone of solution of p-chloranil in dioxane.

Future Work

Future work will involve purification of the sample materials, electrical conductivity measurements, and studies of the nature of the photo-induced chemical reactions. Vapor-phase chromatography (VPC) has been used to determine the degree of purity of some of the solvents. As preparative VPC will soon be available, which will be used in conjunction with a spinning-band distillation column to purify the solvents. Photoconductivity measurements will be carried out with the aim of being able to differentiate between electronic and ionic conductivity. Preliminary studies are under way, but a high-frequency bridge technique will certainly be necessary to demonstrate electronic conductivity. Finally, further kinetic studies of the rise and decay times of the photoinduced ESR signals, together with analyses of the reaction products, should help to elucidate the nature of the chemical reactions occurring in the solutions.

7. FLUORESCENCE OF ORIENTED DYE-MACROMOLECULE COMPLEXES--THEORETICAL STUDY

Gilbert Weill

A recent experiment by Lerman[1] on DNA-quinacrine complexes shows the interest of the study of the variation in fluorescence intensity due to the action of an orientating field on a dye-macromolecule complex to determine the mode and the geometry of the dye binding. This experiment, however, has been interpreted on a purely qualitative basis, involving a number of assumptions that will generally not be valid in the large class of chromophore-macromolecule complexes of biological interest. Therefore, a theoretical calculation of the intensity changes has been undertaken, and the result, valid for a more general model, will permit a more quantitative discussion of the precited experiment.

Choice of the Model

In this first step the calculation is made for a planar dye molecule which can be bound with its plane either parallel or perpendicular to the polymer axis. All the relaxation times of the macromolecule are assumed to be large in comparison with the fluorescence lifetime, so that the angle between the absorption transition moment μa and the emission transition moment μe is a result only of the electronic structure of the dye, and can be evaluated from measurements of the polarization of fluorescence of the unoriented system. (This can be experimentally checked by varying the viscosity or the temperature.)

Set of Coordinates

Let Ouvw be a coordinate system with Ow as direction of the polymer axis, Ouw as the plane of the dye when parallel to the axis, and Ouv the plane of the dye when perpendicular to the polymer axis. The angular coordinates of μa and μe are given in terms of α and of the angle Ow, $\mu a = \chi$. (Table 7-I.) A second system of axes is determined by the direction of propagation of the light, OX, and the directions OY and OZ of the electric vector of the so-called "vertically" and "horizontally" polarized light. The angular coordinates of Ouvw in the system OXYZ are functions of the three Eulerian angles ϕ, γ, θ. (Table 7-II.)

Polarized Components of the Fluorescence

Observing the fluorescence along direction OY and using an analyzer analyzing along the directions OZ and OX combined with a polarizer polarizing along the directions OZ and OY leads to the definition of four polarized components of the fluoresced light, each proportional to the mean value of the product of the square of the projection of μa along one of the axes OZ and OY by the square of the projection of μe along one of the axes OZ and OX, the mean value being taken on all the orientations of Ouvw:

$$I_{yx} = \text{Constant} \langle \mu^2_{ay} \mu^2_{ex} \rangle \qquad I_{yz} = \text{Constant} \langle \mu^2_{ay} \mu^2_{ez} \rangle$$
$$I_{zx} = \text{Constant} \langle \mu^2_{az} \mu^2_{ex} \rangle \qquad I_{zz} = \text{Constant} \langle \mu^2_{az} \mu^2_{ez} \rangle$$

1. L. S. Lerman, Proc. Natl. Acad. Sci. U. S. 49, 94 (1963).

Table 7-I. Directions of the transition moments.

		Ou	Ov	Ow
Dye parallel	μa	$\sin \chi$	0	$\cos \chi$
	μe	$\sin (\chi + \alpha)$	0	$\cos (\chi + \alpha)$
Dye perpendicular	μa	1	0	0
	μe	$\cos \alpha$	$\sin \alpha$	0

Table 7-II. Orientation of axes bound to the polymer and the dye.

	OX	OY	OZ
Ou	$\cos \phi \cos \theta \cos \gamma - \sin \phi \sin \gamma$	$\sin \phi \cos \theta \cos \gamma + \cos \phi \cos \gamma$	$- \sin \theta \cos \gamma$
Ov	$-\cos \phi \cos \theta \sin \gamma - \sin \phi \cos \gamma$	$-\sin \phi \cos \theta \sin \gamma - \cos \phi \cos \gamma$	$\sin \theta \sin \gamma$
Ow	$\cos \phi \sin \theta$	$\sin \phi \sin \theta$	$\cos \theta$

If we apply an orientating field along the Z axis, the orientation of Ouvw will no longer be random, but will be given by a distribution function of the general type

$$dW(\theta, \phi, \gamma) = K \exp(U(\theta)/kT) \sin \theta \, d\theta \, d\phi \, d\gamma,$$

where $U(\theta)$ is the potential energy of the molecule in the field and depends on the type of orientation. Generally U is independent of ϕ and γ, and the polarized components can be calculated as a function of two mean functions of the angle of orientation--for example, $\sin^2 \theta$ and $\sin^4 \theta$. The result is given in Table 7-III. It is easy to verify, assuming random orientation, that these expressions give the classical value of the polarization of fluorescence in infinitely viscous medium:

$$P_0 = (3 \cos^2 \alpha - 1)/(\cos^2 \alpha + 3).$$

The expressions should allow a complete interpretation of the variation of intensity produced by an electric or hydrodynamic field and, completed by measurements of the variation of the dichroic ratio produced by orientation of μa alone, a better determination of the binding of the dye as well as the mechanism of orientation of the macromolecule.

Table 7-III. Expressions for the polarized components of the fluoresced light.

Dye parallel

Time corresponding column	I_{yx}	I_{yz}	I_{zx}	I_{zz}
$\sin^2\chi \sin^2(\chi+a)$	$(1/64)(8+3\sin^4\theta-8\sin^2\theta)$	$(1/16)(4\sin^2\theta-3\sin^4\theta)$	$(1/16)(4\sin^2\theta-3\sin^4\theta)$	$(3/8)\sin^4\theta$
$+\cos^2\chi \cos^2(\chi+a)$	$(1/8)\sin^4\theta$	$(1/2)(\sin^2\theta-\sin^4\theta)$	$(1/2)(\sin^2\theta-\sin^4\theta)$	$1+\sin^4\theta-2\sin^2\theta$
$+\sin^2\chi \cos^2(\chi+a)$	$(1/16)(4\sin^2\theta-\sin^4\theta)$	$(1/4)(2+\sin^4\theta-3\sin^2\theta)$	$(1/4)\sin^4\theta$	$(1/2)(\sin^2\theta-\sin^4\theta)$
$+\cos^2\chi \sin^2(\chi+a)$	" " "	$(1/4)\sin^4\theta$	$(1/4)(2+\sin^4\theta-3\sin^2\theta)$	" " "
$+\sin\chi\cos\chi \sin(\chi+a)\cos(+)$	$(-4/16)\sin^4\theta$	$-\sin^2\theta+\sin^4\theta$	$-\sin^2\theta+\sin^4\theta$	$2(\sin^2\theta-\sin^4\theta)$

Dye perpendicular

	I_{yx}	I_{yz}	I_{zx}	I_{zz}
$\cos^2 a$	$(1/64)(8+3\sin^4\theta-8\sin^2\theta)$	$(1/16)(4\sin^2\theta-3\sin^4\theta)$	$(1/16)(4\sin^2\theta-3\sin^4\theta)$	$(3/8)\sin^4\theta$
$+\sin^2 a$	$(1/64)(24+\sin^4\theta-24\sin^2\theta)$	$(1/16)4\sin^2\theta-\sin^4\theta)$	$(1/16)(4\sin^2\theta-\sin^4\theta)$	$(1/8)\sin^4\theta$

Comparison with the Experimental Results on the DNA-Quinacrine Complex

The polarization of fluorescence in the unoriented system suggests that the dye has two absorption transitions, one corresponding to $a = 0^\circ$, the other to $a \approx 90^\circ$. Lerman therefore was able to study qualitatively the change of intensity produced by a complete orientation for those two absorptions and three dispositions of the dye and compare them with the sign of variation obtained experimentally for a partial orientation. The three dispositions can be expressed in terms of χ and of the position of the plane of the dye in respect to the polymer axis. The sign of the variation of intensity with orientation comes directly from the differentiation of the expressions in Table 7-III. In some cases the result depends on the mechanism of orientation and on the degree of orientation, and the result agrees with Lerman's prediction only for a large orientation $[d(\sin^2\theta - \sin^4\theta)/d\theta < 0]$. The results are summarized in Table 7-IV.

Table 7-IV. Prediction of change of intensity upon orientation.

Lerman notation						
TL	$\chi = 90^\circ$	$d(I_{yx})$	$d(I_{zz})$	$\chi = 0^\circ$	$d(I_{yx})$	$d(I_{zz})$
	$a = 0^\circ$	+	-	$a = 90^\circ$	-	- (or +)
TS	$\chi = 0^\circ$			$\chi = 0^\circ$		
	$a = 0^\circ$	-	+	$a = 90^\circ$	-	- (or +)
P	Whatever a dye perpendicular	+	-			

The experimental values given by Lerman are

λ excitation	dI_{yx}/I_{yx}	dI_{zz}/I_{zz}	dI_{yz}/I_{yz}	dI_{zx}/I_{zx}
4500 Å ($a = 0^\circ$?)	+0.35	-0.29	-0.11	-0.10
3000 Å ($a = 90^\circ$?)	±0.24	-0.26	0.12	-0.10

These data fit the prediction of the arrangement P. As the polarization of fluorescence of the unoriented solution is compatible, however, with values of $a = 30^\circ$ and $a = 60^\circ$ for the two transition--which is likely for an unsymmetrical dye like quinacrine--these values have been introduced in the expressions of the intensity for the dye parallel to the polymer axis. In the absence of information on the degree of orientation, evaluations are difficult, but no set of signs of variation similar to the experimental results has been obtained. As there are additional reasons to believe in the arrangement P (as defined in Table 7-IV),[2,3] we have tried to derive some other results from our theoretical expressions.

2. L. S. Lerman, J. Mol. Biol. 3, 18 (1961).
3. V. Luzzati, F. Masson, and L. S. Lerman, J. Mol. Biol. 3, 639 (1961).

(a) dI_{zz}/I_{zz} must be independent of α,

(b) $dI_{yz}/I_{yz} = dI_{zx}/I_{zx}$,

(c) dI_{yz} is negative for high degrees of orientation but can be positive for low degrees of orientation when α is 0°,

(d) dI_{yz}/I_{yz} depends generally on α,

(e) dI_{xx}/I_{xx} depends strongly on α. For high degrees of orientation the variation must be greater for $\alpha = 0^\circ$ than for $\alpha = 90^\circ$.

The predictions (a), (b), (c), and (e) are verified by Lerman's experimental results, and bring additional evidence for the perpendicular arrangement. Experiments are now planned on a similar system to measure the variations as a function of the degree of orientation and to try a quantitative verification of the theoretical formulae.

8. FORMATION OF ADENINE BY ELECTRON IRRADIATION OF METHANE, AMMONIA, AND WATER

Cyril Ponnamperuma,* Richard M. Lemmon, and Melvin Calvin

Previous reports from this Laboratory described the nature of some of the compounds formed on the electron irradiation of gaseous mixtures of methane, ammonia, and water.[1,2] The principal purpose of that research was to look for evidence for the production of nucleic acid components under "primitive earth atmosphere" conditions. Amino acids, the constituents of proteins, are already known to be formed on electric-discharge or accelerated-electron irradiations of these gaseous mixtures. The earlier reports[1,2] presented proof of the synthesis of urea and some evidence for the possible synthesis of an important nucleic acid constituent--namely, adenine. The work described here shows that (a) adenine is indeed a product of the electron irradiation of a mixture of methane, ammonia, and water, (b) there is an inverse relationship between the amount of adenine synthesized and the amount of hydrogen gas present, and (c) of the five nucleic-acid bases, adenine is the one most readily synthesized under the "prebiotic" earth conditions.

Experimental Procedure

Mixtures of methane-C^{14}, ammonium hydroxide (4N), and, in some experiments, hydrogen were irradiated in the glass apparatus (volume approx 750 ml) shown in Fig. 8-1. Four separate experiments were performed.

Twenty ml of 4N NH_4OH and two or three boiling chips (SiC) were introduced into the irradiation tube. The flask B was cooled to -78° and the tube evacuated. Twenty-five mmoles of $C^{14}H_4$ (containing 0.5 mC; source: Tracerlab, Inc. and New England Nuclear Corp.) was introduced, followed by nonlabeled methane (12 mmoles) until the pressure in the tube was 300 mm. During the methane addition, care was exercised to exclude all air from inlet tubes. In two experiments, no H_2 was used. In a third experiment 50 mm of H_2 was added to the above mixture; in a fourth experiment 100 mm of H_2 was introduced into the irradiation tube.

During the irradiations the irradiation tube was kept in a horizontal position. The electrons entered the tube through the concave end window, which was larger in diameter than the cross section of the electron beam. In all experiments, the electrons were delivered by a linear electron accelerator. These electrons had an energy of 4.5 MeV and were delivered in 60 pulses per second, each pulse lasting 6 μsec. The integrated dose rate was 18 μA, and the time of irradiation was 45 minutes. The current used during this time was 0.0486 coulombs. Cobalt glass dosimetry at the center

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1. Christof Palm and Melvin Calvin, J. Am. Chem. Soc. 84, 2115 (1962).

2. Christof Palm and Melvin Calvin, in Bio-Organic Chemistry Quarterly Report, UCRL-9900, October 1961, p. 51.

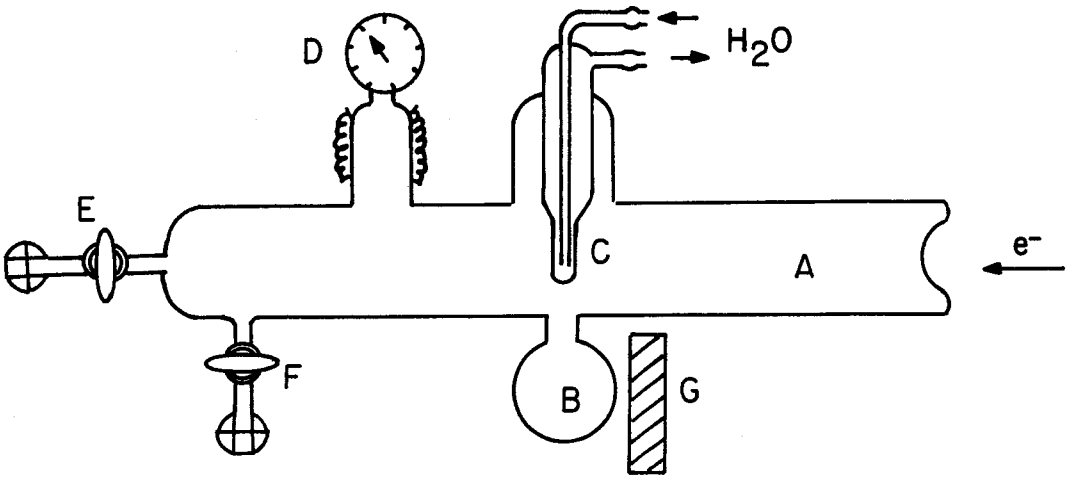

Fig. 8-1. A: Irradiation tube; dimensions: length, 50 cm, diameter 4 cm.
B: flask containing NH_4OH. C: cold-finger condenser.
D: pressure and vacuum gauge. E and F: vacuum-seated stopcocks. G: lead shield.

of a similar irradiation tube indicated absorption of 1.5×10^4 rads per microcoulomb.[4] Our total energy absorption was therefore about 7×10^8 rads, or 7×10^{10} ergs/gram.

The liquid in flask B (Fig. 8-1) was boiled during the irradiations; heat was supplied by two infrared lamps. The irradiations took place in both the gas and the liquid (on the cold-finger surface) phases. The boiling caused a continuous washing back into B of the condensate on C. During the irradiation the pressure in the tube rose to 1.0 to 1.5 atmospheres.

At the end of the reaction the liquid in B was removed. The entire tube was washed with about 20 ml of water and the washings were added to the reaction products. Volatile products were discarded.

Analyses for the nonvolatile products were carried out on aliquot portions by means of paper chromatography on oxalic acid–washed Whatman No. 4 paper. An aliquot portion of the product was placed on the paper together with carrier adenine. The paper was developed with n-propanol-16 N NH_4OH-water (6:3:1 by volume) in one direction and n-butanol–glacial formic acid–water (77:10:13 by volume) in the other. Two other solvent systems were also used: n-butanol-water (86:14 by volume) and isopropanol-2 N HCl (65:35 by volume). The distribution of radioactivity on the chromatograms was recorded by autoradiography with x-ray film. The adenine was located on the chromatograms through the use of shadowgrams.[5,6] The percentage of adenine formed from CH_4 was determined by eluting the adenine spots from the chromatograms and measuring the activity by liquid scintillation counting.

Other series of chromatograms were run in which the other purine and pyrimidines commonly found in the nucleic acids (guanine, cytosine, uracil, and thymine) were used as carriers.

Results and Discussion

The following conditions were all held constant through the four experiments: CH_4 pressure (300 mm), $C^{14}H_4$ activity (0.5 mC), NH_3 and H_2O pressure (a total of about 1.5 atm, from 20 ml of 4N NH_4OH at about 100°), and energy absorption (about 7×10^{10} ergs). The one variable was the amount of added H_2. The amount of adenine produced as an apparent function of this variable is shown below.

In all four experiments, and in all four paper chromatographic solvent systems, there was perfect coincidence in both position and shape between the inactive carrier adenine (shadowgrams) and one of the radioactive-product spots (x-ray film darkening).

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4. J. Oró, in Bio-Organic Chemistry Quarterly Report, UCRL-10634, Jan. 1963, p. 102.
5. J. D. Smith and R. Markham, Biochem. J. 45, 294 (1949).
6. C. A. Ponnamperuma, The Radiation Chemistry of Nucleic Acid Constituents (Thesis), UCRL-10053, June 1962, p. 36.

Experiment	1	2	3	4
H_2 pressure (mm)	0	0	50	100
$C^{14}H_4$ converted to adenine-C^{14}(%)	0.012	0.010	0.002	0.001

The results of this investigation clearly establish adenine as a product of the irradiation of methane, ammonia, and water. Furthermore, it appears that the production of adenine is enhanced by the absence of H_2. This is not surprising, since methane carbon must be oxidized in order to appear finally in purines and amino acids. The hydrogen would be expected to interfere with the oxidative processes. In our system, the principal species affecting the oxidations are probably OH and NH_2 radicals, and these radicals would revert to the starting materials (water and ammonia) on reaction with hydrogen:

$$\cdot OH + H_2 \rightarrow H_2O + H\cdot \ ,$$

$$\cdot NH_2 + H_2 \rightarrow NH_3 + H\cdot \ .$$

The first reaction is energetically favored, since the H-H bond energy is 104.2 ± 0.1 kcal and the HO-H bond energy is 119 ± 1 kcal.[7] The second reaction is slightly unfavored (H_2N-H bond energy is 103 ± 1 kcal) and may not occur. Another possible way in which H_2 might interfere with the production of purines would be to cause methyl radicals to revert to methane through the reaction $\cdot CH_3 + H_2 \rightarrow CH_4 + \cdot H$. Here the bond energies are very similar: H-CH_3 (104 ± 1 kcal), H-H (104.2 ± 0.1 kcal).[7] In any event, the high concentration of organic matter on the prebiotic earth probably arose only at a time when most of the hydrogen had escaped from the atmosphere. Other results of our present work have indicated that there is also an inverse relationship between the presence of hydrogen and the synthesis of amino acids.

No guanine, cytosine, uracil, or thymine was detected on any of our chromatograms. Any one of these bases would have been detected if it had been present in one-hundredth the amount of the adenine. The apparent preference for adenine synthesis may be related to adenine's multiple roles in biological systems. Not only is it a constituent of both DNA and RNA, but it is also a unit of many important cofactors--for example, ATP, ADP, DPN, TPN, FAD, and coenzyme A. In addition, molecular orbital calculations have shown that, of the biologically important purines and pyrimidines, adenine has the greatest resonance energy.[8,9] This would not only make adenine's synthesis more likely, but would, in addition, confer radiation stability upon it. Thus, after formation, the adenine would be more likely than the other bases to survive the radiation fields of our experiments.

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7. J. A. Kerr and A. F. Trotman-Dickenson, Handbook of Chemistry and Physics, 44th Ed. (Chemical Rubber Publishing Co., Cleveland, Ohio, 1962-63), pp. 3519-20.

8. B. Pullman and A. Pullman, Nature 196, 1137 (1962).

9. B. Pullman and A. Pullman, in Comparative Effects of Radiation (John Wiley and Sons, New York, 1960), pp. 111-112.

9. UPTAKE OF ORGANIC COMPOUNDS BY PLANARIANS

Edward L. Bennett, Marie Hebert, and M. Calvin

Recently there have been a number of suggestions that memory must have a molecular basis. Hydén has proposed that ribonucleic acid is intimately associated with learning.[1, 2] Other possible mechanisms have been discussed by Briggs and Kitto,[3] and by Smith.[4] To date, however, the results of the few experiments with higher animals to demonstrate a molecular basis for learning have certainly been open to several interpretations.

Recent experiments with planarians suggest that this primitive animal may be useful for investigating the possibility of a molecular basis of learning.[5-7] Several investigators have shown that planarians can be trained. Usually this training consists of conditioning the planarian to respond to light, which is followed by a mild electric shock. However, other forms of learned behavior have been demonstrated.[5] McConnell, Jacobson, and Kimble showed that the planarians regenerating from either the head or tail of a conditioned planarian cut in half were capable of memory storage or, in the parlance of psychologists, exhibited "savings."[5, 6] More recently, Corning and John have reported that savings could be demonstrated in planarians that had regenerated in ribonuclease from heads, but not in planarians that had regenerated in ribonuclease from tails.[5, 7]

As a prelude to a projected study of possible mechanisms of the retention of a conditioned response or "savings" in the planarian, we have initiated a study of the uptake and utilization of numerous organic molecules into the planarian. One ultimate objective is to utilize the labeled macromolecules formed from small precursor molecules for studying the distribution and retention of different classes of macromolecules when worms either regenerated or are ingested by other planarians. Ultimately, it is hoped, various classes of molecules or even specific molecules may be isolated and be shown to transfer "memory."

The uptake of (a) purines, pyrimidines, and nucleosides, (b) amino acids, (c) fatty acids, and (d) carbohydrates by planarians has been compared. Of these compounds, fatty acids are taken up most extensively. Butyrate-2-^{14}C and valerate-2-^{14}C are taken up more rapidly than the lower fatty acids, and form at least 10 to 15 compounds. Propionate-2-^{14}C, which is also extensively utilized by planarians, is lost only slowly. Extensive transfer and retention of radioactivity can be shown when nonradioactive planarians are fed planarians previously labeled with propionate-2-^{14}C.

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1. H. Hydén, Sci. Am. 205 (6), 62 (1961).

2. H. Hydén and E. Egyhazi, Proc. Natl. Acad. Sci. U.S. 48, 1366 (1962).

3. M. H. Briggs and G. B. Kitto, Psychol. Rev. 69, 537 (1962).

4. C. E. Smith, Science 138, 889 (1962).

5. J. B. Best, Sci. Am. 208 (2), 55 (1963).

6. J. V. McConnell, A. L. Jacobson, and D. P. Kimble, J. Comp. Physiol. Psychol. 52, 1 (1959).

7. W. C. Corning and E. R. John, Science 134, 1363 (1961).

Of the nucleic acid precursors tested, adenine and cytosine exhibited the greatest uptake. The uptake of adenine (and presumably of the other organic compounds tested) is concentration-dependent. Conversion of adenine to 5'-adenylic acid and nucleic acid adenine and nucleic acid guanine has been demonstrated. The amino acids and carbohydrates are taken up only slowly.

Methods

Typically, the planarians were placed in the radioactive solution contained in a 10-ml or 20-ml beaker and stored in a dark cupboard. The "concentration" of planarians was 3 or 4 per ml of solution, and the concentration of the radioactive compound was generally 2.5 μmoles/ml for the nucleic acid precursors or 10 μmoles/ml for the other compounds tested. Spring water (Alhambra) was used, and the pH was adjusted to 7 after the compound was dissolved. Prior to analysis, a planarian was removed from the radioactive solution and passed through three successive rinses of water in a spot plate. The following basic fractionation procedures were followed:

1. Trichloroacetic acid (TCA) method

An individual planarian was homogenized in 500 μl of distilled water, by use of a micro Teflon-glass homogenizer (25 μl of the homogenate was removed for a determination of the total radioactivity present), 100 μl of 50% TCA was added to the homogenate, and the homogenate was centrifuged. The precipitate was washed twice with 500 μl of 10% TCA, and the activity of the combined supernatants (TCA-soluble fraction) was determined by counting a 100-μl aliquot. Subsequently, in some experiments, the TCA was removed by continuous ether extraction, and the activity in the aqueous phase was redetermined. The residue in the homogenizer was extracted twice with 500 μl of 95% ethanol and then dissolved by warming overnight at 37° with 50 μl of 1 N KOH. The basic solution was then diluted to 500 μl and heated (80°) for 2 to 3 hr. (The ethanol extraction was omitted in some preliminary experiments.) Aliquot portions were taken of the ethanol extracts and the KOH-hydrolyzed residue for determination of radioactivity. An approximate measure of the size of the worm has usually been made by determining the protein content of an aliquot of the KOH-soluble residue, utilizing the Folin-Wu color reaction.[8] When protein determinations have been made, the results have been expressed in terms of mμM uptake per 100 γ protein or 1 mg planarian. A planarian is about 10% protein, and those used normally weighed 2 to 4 mg.

2. Ethanol extractions

In a later series of experiments, the fractionation procedure has been patterned after that commonly used in photosynthesis experiments. The planarian has been homogenized and extracted twice with 500 μl of 80% ethanol-20% H_2O at 50°, then twice with 500 μl of 30% ethanol-70% H_2O. In one series of experiments acetone-water mixtures were also used.

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8. O. H. Lowry, N. H. Rosenbrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193, 265 (1951).

The residue has been dissolved by warming with 50 µl of 1 N KOH or by heating overnight at 80° with 100 µl of 6 N HCl. In either case, the solution was diluted to 500 µl with H_2O prior to sampling. No protein determinations were made after acid hydrolysis.

Scintillation counting has been used to determine the radioactivity. "Scintillator 2," which consists of 5.0 g of 2, 5-diphenyl oxazole (PPO), 100 mg of 1, 4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP), 50 g naphthalene, 400 ml of p-dioxane, 250 ml of ethanol, diluted to 1 liter with toluene, has been employed. Normally, samples have been counted shortly after addition to the scintillator solution in order to minimize a small (maximum 10%) decrease in activity due to settling of particulate material in some samples. The efficiency was routinely determined by the addition of toluene-^{14}C (or toluene-^{3}H in the few experiments with tritiated compounds) and was normally 60 to 65% for ^{14}C.

Extracts from the planarians have been chromatographed two-dimensionally on Whatman No. 1 filter paper in phenol-H_2O and then in butanol–propionic acid–H_2O. Radioactive compounds have been located by radioautography with Kodak x-ray film, and the distribution of radioactivity has been estimated by counting the principal radioactive areas with a Mylar-window GM tube. Carrier amino acids have been located by spraying with ninhydrin. In experiments with nucleic acid precursors, 60% propanol–30% ammonium hydroxide–10% H_2O (by volume) has been substituted for phenol-H_2O and the location of carrier compounds has been determined under uv light.

Results

Effect of concentration on uptake

The uptake of adenine and thymine is dependent on the external concentration (Table 9-I). In this experiment, approximately 7 to 10% of the total adenine-^{14}C activity taken up by the planarians was found in the RNA-DNA fraction (TCA-insoluble fraction). Paper chromatograms of the material extracted by 10% TCA indicated that only 5 to 10% of the radioactivity was unmetabolized adenine; a major portion was 5'-adenylic acid and higher phosphorylated derivatives. Hypoxanthine was another principal constituent present. 3'-Adenylic acid and 3'-guanylic acid were found in alkaline hydrolysates of the TCA-insoluble material, indicating unequivocally that RNA had been formed from adenine.

Thymine-2-^{14}C uptake was also dependent on the external concentration; however, the increase in uptake was found principally in the TCA-soluble fraction, and the external concentration influenced uptake into the TCA-insoluble (RNA-DNA) fraction only slightly.

The effect of concentration on the uptake of other compounds has not been tested, but presumably the uptake of most or all organic molecules is concentration-dependent.

Table 9-I. Effect of concentration on the uptake of adenine-8-^{14}C and thymine-2-^{14}C into planarians.

Compound	Concentration (μM/ml)	mμM in aqueous homogenate of planarian	mμM in 10% TCA-soluble fraction	mμM in 10% TCA-insoluble fraction
Adenine-8-^{14}C	0.134	1.3	1.0	0.08
	0.30	2.8	2.1	0.18
	0.56	5.0	4.3	0.29
	1.21	10.7	7.7	0.78
Thymine-2-^{14}C	0.28	0.14	0.06	0.04
	0.56	0.28	0.15	0.05
	1.15	1.3	0.63	0.07

Three planarians (Dugesia dorotocephala) (Dahl Laboratory, Berkeley) were placed in 1 ml of a solution of adenine-8-^{14}C (1.34 μC) or thymine-2-^{14}C (0.64 μC) for 2 days and then analyzed. The size of these planarians was not determined, and results are expressed per planarian.

Rate of uptake and retention of adenine-8-^{14}C

Two experiments, sumarized in Table 9-II, indicated that the total uptake of adenine increased with time. The radioactivity was not rapidly lost upon transfer of planarians to a solution devoid of external adenine. The low uptake found at 2 hours is evidence that the passage of a planarian through three successive washes in a spot plate was sufficient to remove externally adsorbed radioactivity. The proportion of activity in the RNA-DNA fraction (TCA-insoluble fraction) increased when planarians were removed from the adenine-^{14}C solution. This is consistent with the concept that material from the TCA-soluble (nucleotide) pool is utilized for nucleic acid formation by planarians. Paper chromatograms of the adenine solution in which planarians had been soaking for 2 days showed no formation of radioactive impurities. The TCA-soluble extract of the planarians exposed to adenine for 2 days contained approximately 50% of the activity as 5'-AMP, ADP, and ATP, and 7% as adenine, and approximately 20% has been tentatively identified as hypoxanthine.

Uptake of pyrimidines and nucleosides

The utilization of the pyrimidines, uracil, and cytosine, and the nucleosides, uridine, cytidine, and adenosine, were compared (Table 9-III). Uracil was taken up most extensively into the 10% TCA-soluble fraction, and a maximum of 10% of the uracil was incorporated into the TCA-insoluble fraction. A larger proportion of each of the other compounds tested went into the TCA-insoluble fraction, with cytosine exhibiting the maximum incorporation into the TCA-insoluble (presumably nucleic acid) fraction. A comparison of the data obtained from this experiment with data from previous experiments using adenine-8-^{14}C showed that 5 to 20 times as much adenine was taken up into the TCA-soluble fraction as of any of the other compounds compared. Cytosine apparently exhibited 5 to 10 times as great uptake into

Table 9-II. Rate of uptake and retention of adenine-^{14}C in planarians.

Time of soaking in ^{14}C	Days in H_2O	mμM adenine in TCA-soluble fraction	mμM adenine in TCA-insol. fraction	% Total
Experiment 1[a]				
2 hr		0.68	0.05	7
6 hr		1.83	0.08	4
1 day		4.7	0.35	7
2 days		7.8	0.7	8
2 days	1	4.8	1.48	24
Experiment 2[b]				
4 hr		1.9	0.03	2
1 day		7.0	0.22	3
2 days		10.5	0.72	6
2 days	1	7.0	1.1	14
2 days	2	6.1	2.2	27
2 days	4	4.0	0.8	16

a. Fourteen planarians (Dugesia dorotocephala) were placed in a 3-ml solution containing 2.7 μmoles of adenine-8-^{14}C/ml. After 2 days, remaining planarians were transferred to a solution of spring water. Average results from the two planarians taken at each sampling time presented.

b. Same procedure, except 16 Dugesia tigrina used initially and the concentration of the adenine-8-^{14}C was 2.5 μmoles/ml.

Table 9-III. Comparison of rate of uptake of uracil-2-^{14}C, uridine-2-^{14}C, cytosine-^{3}H, cytidine-^{3}H, and adenosine-^{3}H [a] (mμM incorporated/mg planarians).

Time in radioactive solution	Uracil-2-^{14}C			Uridine-2-^{14}C			Cytosine-^{3}H			Cytidine-^{3}H			Adenosine-^{3}H		
	TCA-soluble	TCA-insol.	% Total in TCA-insol.	TCA-soluble	TCA-insol.	% Total in TCA-insol.	TCA-soluble	TCA-insol.	% Total in TCA-insol.	TCA-soluble	TCA-insol.	% Total in TCA-insol.	TCA-soluble	TCA-insol.	% Total in TCA-insol.
3 hr	0.090	0.002	2	0.05	0.009	15	0.30	0.12	28	0.05	0.005	9	0.03	0.006	17
1 day	0.46	0.03	6	0.09	0.027	23	0.50	0.25	33	0.11	0.025	18	0.09	0.03	25
2 days	0.55	0.03	5	0.25	0.07	22	0.25	0.37	59	0.16	0.09	36	0.06	0.05	45
3 days	0.65	0.07	10	0.23	0.08	26	0.23	0.38	62	0.21	0.09	30	0.08	0.06	43
3 days in 1 day out	--	--	--	0.21	0.12	36	0.10	0.32	76	0.12	0.05	29	0.07	0.05	48

a. All radioactive compounds were obtained from New England Nuclear Corporation. Fifteen Dugesia dorotocephala were placed in a 10-ml beaker containing 10.4 to 10.9 μmoles of the radioactive compound dissolved in 4 ml of spring water and pH was adjusted to 7.0. Approximately 30 μC of a ^{14}C-labeled compound or 200 μC of a ^{3}H-labeled compound was used. Two planarians were sampled at each time interval. Results are expressed in terms of mμM of compound utilized per mg planarians.

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the TCA-insoluble fraction as adenine. The low degree of uptake into the TCA-soluble fraction of precursors other than adenine may be a reflection of pool size. In higher animals, adenine derivatives are typically the most abundant. Since the cytosine pool is small, the activity in the nucleic acid fraction from a cytosine-labeled planarian does not increase when it is placed in a nonradioactive medium. The activity in the nucleic acid fraction in a worm given adenine does increase when the planarian is removed from the radioactive medium. At present the relative incorporation of adenine and cytosine into nucleic acids has not been firmly established. One experiment in which the external concentration was 10 μM/ml indicated that cytosine was utilized 5 to 10 times as well as adenine, whereas several experiments at 2.5 μM/ml suggest more nearly equal utilization of these two nucleic acid derivatives.

Incorporation of amino acids into planarians

The incorporation of glycine-2-^{14}C, DL-alanine-2-^{14}C, DL-serine-3-^{14}C, L-methionine-methyl-^{14}C, L-valine-4,4'-^{14}C, and L-leucine-3-^{14}C was compared in one experiment. The concentration used was 10 μmoles/ml in each case. Samples of the solutions in which the planarians were soaking were removed daily and the radioactive purity checked by chromatography and radioautography. The valine and methionine contained 20 to 30% impurity initially; the other amino acids were chromatographically pure. The impurity in the methionine increased to 50% after 3 days. Chromatograms of the amino acid solutions made after soaking the planarians for 2 or 3 days indicated that a large proportion (up to 30 to 40%) of the radioactivity now remained at the origin.

The incorporation of the radioactive amino acids into the TCA-soluble fraction is summarized in Fig. 9-1a, and the incorporation into the TCA-insoluble fraction is presented in Fig. 9-1b. Methionine has been omitted from the figures, but its incorporation roughly paralleled that of alanine. The amount of radioactive amino acid taken up increased with time. The increase was probably approximately linear when allowance was made for the fact that each point is the average of data obtained from only two planarians, and the "spread" was often 20 to 30%. There were no great differences in uptake of the amino acids into the soluble pool (except for valine, which was initially impure). The radioactivity decreased relatively rapidly from the soluble fraction when planarians were removed from the radioactive solutions. The incorporation into the TCA-soluble fraction was 1.5 to 2.5 mμmoles/mg planarians at 2 days, compared with 8 to 12 mμmoles for adenine under comparable conditions.

The radioactivity of the TCA-insoluble fraction (presumably primarily in protein) increased up to 2 days when the planarians were soaking in radioactive amino acids, and this fraction did not decrease greatly after the planarians were removed and placed in water for 5 days. Approximately 40% of the activity in the planarians at 2 days was 10% TCA-insoluble.

Uptake of fatty acids

The uptake of the fatty acids, sodium formate-^{14}C, sodium acetate-2-^{14}C, sodium propionate-2-^{14}C, sodium butyrate-2-^{14}C, and sodium valerate-2-^{14}C into Dugesia tigrina into the 10% TCA-soluble and the 10%

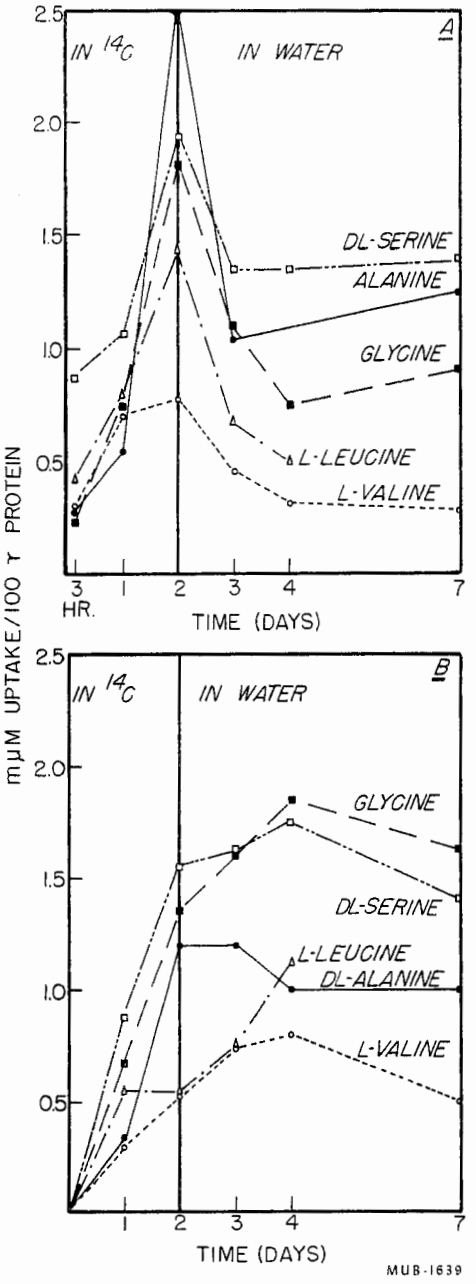

Fig. 9-1. Rate of uptake and retention of labeled glycine, DL-alanine, DL-serine, L-valine, and L-leucine into 10% TCA-soluble and 10% TCA-insoluble material by Dugesia tigrina (South Carolina Biological Supply Co.). The planarians were in the radioactive amino acid solution (10 μM/ml) for periods up to 2 days and then placed in fresh water for periods up to 5 days. The results are expressed in terms of muM uptake/100 γ protein or per mg planarian. Fig. 1a presents data for the 10% TCA-soluble material; Fig. 1b for the 10% TCA-insoluble material.

TCA-insoluble fraction is summarized in Fig. 9-2. An ethanol extraction was made after the 10% TCA extractions, but since it contained a relatively small amount of radioactivity, the results are not presented.

The fatty acids have been taken up much more extensively and rapidly than either the amino acids or nucleic acid constituents. The higher the fatty acid in molecular weight, the more extensive the uptake. Less activity was found in the 10% TCA-insoluble material than was found in the 10% TCA soluble fraction. The activity decreased only slowly in each fraction when the planarians were removed from the radioactive solution. About 50% of the 10% TCA-soluble material was ether extractable after 5 hours' exposure to a radioactive fatty acid, but less than 20% of the activity was extractable after 24 hours' or longer uptake of the fatty acids.

Paper chromatograms of the 10% TCA-soluble fraction were disappointing, since the majority of the activity remained at the origin. However, if this fraction from a planarian exposed to labeled valerate or butyrate were initially subjected to HCl hydrolysis, as many as 15 radioactive compounds were obtained. Four of the compounds have been tentatively identified as glutamic acid, aspartic acid (the major compounds), serine, and alanine. Hammen and Lu have shown the rapid conversion in planarians of propionate-2-^{14}C into α-ketoglutaric acid, succinic, fumaric, malic, citric, isocitric, lactic, and mesoxalic acids, and the formation of aspartic and glutamic acids and threonine from $^{14}CO_2$.[9]

An experiment in which Dugesia tigrina were maintained for 4 weeks in acetate-2-^{14}C or propionate-2-^{14}C indicated that little increase occurred in the total radioactivity incorporated in the 10% TCA-soluble fraction after 1 week in a radioactive solution. The activity in the 10% TCA-insoluble fraction increased up to 2 weeks, at which time apparent "saturation" was reached. The planarians used in these experiments were fed brine shrimp twice weekly.

Uptake of glucose and sucrose

Glucose and sucrose were taken up about equally but were relatively little utilized by Dugesia tigrina. The incorporation into the 10% TCA-soluble fraction was less than 1 mμM/mg planarian at 2 days, or about 25% of that found with acetate. The incorporation into the fraction subsequently extractable by ethanol was about 0.5 mμM/mg, again about 1/4 of that found with acetate. The incorporation into the 10% TCA-insoluble fraction was about 1 mμM/mg, about 1/3 of that found with acetate.

Comparison of fractionation procedures

In the experiments described so far in this report, insolubility in 10% TCA has been the primary criterion used to determine if the radioactive compound has been incorporated into "macromolecules." However, in some of the experiments, notably those utilizing fatty acids as precursors, the radioactivity has not decreased rapidly in the 10% TCA-soluble fraction when the worm has been placed in spring water in the absence of activity.

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9. C. S. Hammen and S. C. Lu, J. Biol. Chem. 237, 2419 (1962).

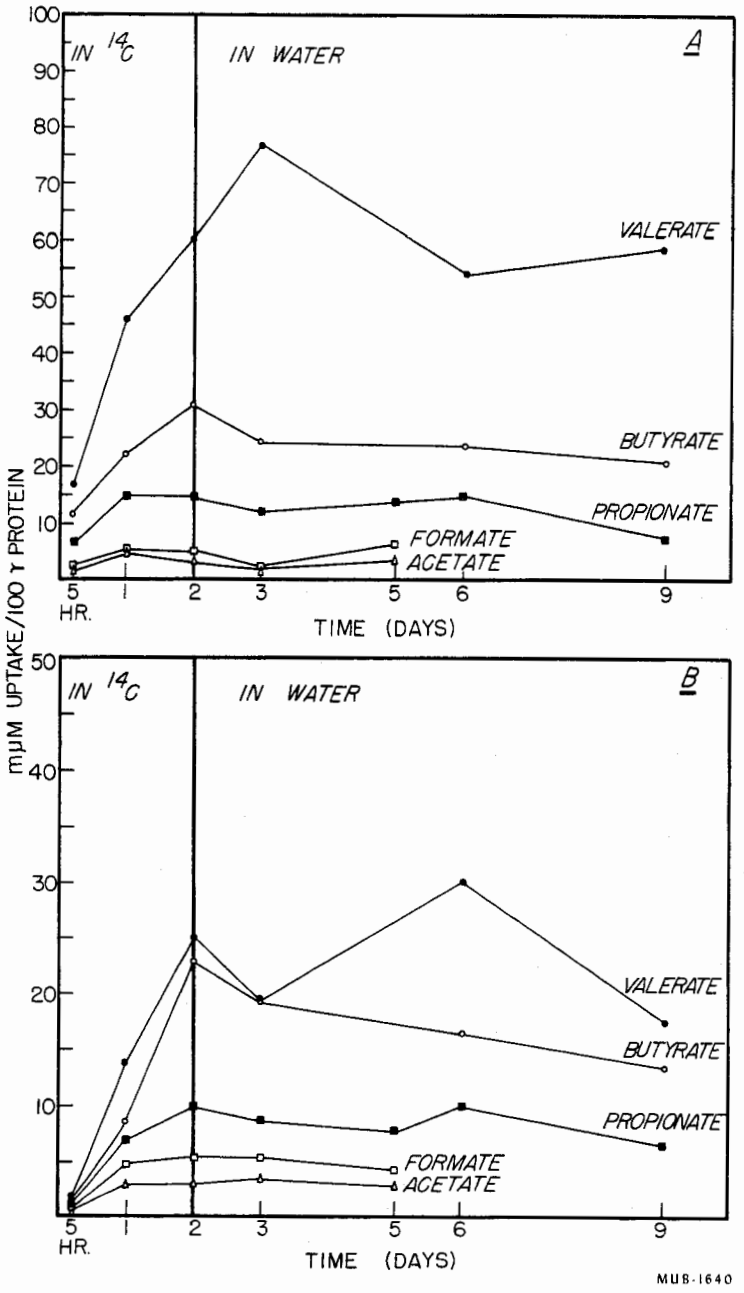

Fig. 9-2. Rate of uptake and retention of five labeled fatty acids by Dugesia tigrina (South Carolina Biological Supply) placed in the ^{14}C-substrate (10 μmoles/ml, pH 7.0). The substrates were all C-2 labeled (except for formate) and 20 to 50 μC of each substrate was used. After 2 days in the radioactive solution, the planarians were removed to fresh nonradioactive water. Fig. 2a presents data for the 10% TCA-soluble material; Fig. 2b for the 10% TCA-insoluble material.

Coupled with the fact that the radioactivity did not move from the origin in chromatograms made of the non-ether-extractable material in the 10% TCA-soluble fraction, this suggested that a portion of this fraction may be composed of molecular weight greater than 1,000 or so.

Therefore, an experiment was carried out in which three methods of fractionation were compared: (a) successive extraction with 80% ethanol-20% H_2O, 30% ethanol-70% H_2O, and digestion of the insoluble residue in HCl; (b) successive extraction in 80% acetone-20% H_2O, 30% acetone-70% H_2O, and an HCl digest of the residue; and (c) successive extractions with 10% TCA, 95% ethanol, and an HCl digest of the residue. The results found when propionate-2-^{14}C was used to label the planarians are summarized in Table 9-IV. At all time intervals, when aqueous ethanol or aqueous acetone was used to extract the worms, a greater percentage of the total activity taken up by a planarian was found in the insoluble fraction, presumably composed of higher-molecular-weight compounds than when the TCA method was used for extraction. The relative increase in insoluble material was approximately 50%. Similar results were obtained when acetate-2-^{14}C, butyrate-2-^{14}C, or valerate-2-^{14}C was employed as substrate. In addition, the results summarized in Table 9-V show the marked increase in the proportion of nonextractable material between (a) 3 hours' uptake, and (b) 2 days' uptake followed by 3 days of incorporation. The data are particularly striking for butyrate-2-^{14}C and valerate-2-^{14}C, for which the proportion of insoluble radioactive material increases from less than 15% to about 75%.

Transfer of radioactivity by feeding labeled planarians

The results summarized in Table 9-VI show that radioactivity is transferred by feeding of radioactive planarians to a nonradioactive planarian. This transferred radioactivity is only slowly lost from the worm that has been fed. More than 60% of this transferred radioactivity is not extracted by ethanol-H_2O. A similar fraction was nonextractable by ethanol-H_2O in the worms used for "food."

Summary

The uptake of numerous organic compounds by Dugesia tigrina has been studied. Fatty acids are rapidly taken up and incorporated into numerous constituents of planarians. Of the precursors for nucleic acids tested, adenine and cytosine are the most efficiently used. Amino acids and carbohydrates are taken up less well. Once taken up, radioactivity is only slowly lost from a planarian. The proportion of the radioactivity classified as insoluble, and therefore existing as macromolecules, depends on the extraction procedure used. Further work is necessary to determine better the size of molecules formed from the labeled precursors. Planarians that are fed radioactive planarians retain and presumably incorporate a major fraction of the ingested activity.

Table 9-IV. Comparison of extraction methods after uptake of propionate-2-^{14}C.[a]

Time of soaking in propionate-2-^{14}C	Ethanol—water			Acetone—water					
	Extracted with			Extracted with			Extracted with		
	80% ETOH 20% H_2O	30% ETOH 70% H_2O	Insoluble	80% acetone-20% H_2O	30% acetone-70% H_2O	Insoluble	10% TCA	95% ethanol	Insoluble
3 hr	54	18	38	50	12	37	64	11	24
1 day	23	12	65	22	11	67	47	9	44
2 days	22	8	69	19	15	66	38	13	50
2 days ^{14}C 3 days H_2O	14	10	76	11	9	80	48	22	30

a. Dugesia tigrina were placed in sodium propionate-2-^{14}C (10 μmoles/ml, pH 7.0). Results are expressed in terms of percentage of total radioactivity in planarian extracted by each series of solvents.

Table 9-V. Distribution of radioactivity after soaking in ^{14}C fatty acids for (a) 3 hours or (b) for 2 days followed by 3 days in H_2O.[c]

Radioactive substrate	Extracted with 80% ethanol-20% H_2O		Extracted with 30% ethanol-70% H_2O		Insoluble	
	(a)	(b)	(a)	(b)	(a)	(b)
Acetate-2-^{14}C	35	26	11	8	54	66
Propionate-2-^{14}C	54	14	8	10	38	76
Butyrate-2-^{14}C	86	14	3	13	11	73
Valerate-2-^{14}C	78	11	5	15	17	73

c. Dugesia tigrina were placed in solutions of the radioactive fatty acids (10 μmoles/ml, 5 to 10 μC/ml, pH 7.0). Planarians were removed and fractionated at 3 h, 1 d, 2 d, and 2 d in ^{14}C-acid, 3 d in water. Only the 3-h and 5-d data are presented here. Planarians fractionated at the intermediate time intervals had a smaller proportion of the total activity present in the 80% ethanol soluble fraction than did the 3-h planarians but more than the 5-d planarians. Extraction of other planarians with 80% acetone-20% H_2O, and then 30% acetone-70% H_2O gave data similar to those for the ethanol fractionation.

Table 9-VI. Transfer of radioactivity by ingestion of planarians previously given propionate-2-^{14}C.[a]

Days after feeding	Extr. by 80% C_2H_5OH-20% H_2O (mμM)	Extr. by 30% C_2H_5OH-70% H_2O (mμM)	Insoluble (mμM)	Total (mμM)
2	6.4	6.0	15.6	28.0
4	4.0	4.4	11.3	19.7
7	2.4	2.5	7.4	12.3

[a] Dugesia tigrina were labeled by soaking in propionate-2-^{14}C for 3 days and then were kept in nonradioactive spring water for 4 days. At this time, a planarian was cut into four approximately equal pieces and fed to four starved D. tigrina. It is estimated that the total activity fed was derived from 30 mμM of propionate-2-^{14}C.

10. THE PLANARIA: ABSORPTION SPECTRUM, CELL DISAGGREGATION, AND STUDIES ON HOMOGENATES

Ning G. Pon and Melvin Calvin

Introduction

The flatworm, planarium, can be trained to respond to external stimuli.[1] If a worm is cut in half transversely, the head portion can regenerate a new tail and the tail section a new head. "Trained" worms, so treated, yield just as much retention of response in both sections as did the "trained" uncut control animals.[2] However, if the regeneration was performed in a weak solution of ribonuclease, the head portion has "savings" of response whereas the tail section does not.[3] Thus it appears that ribonucleic acid is implicated in retention of learning in planaria.

Reduction of memory to a chemical basis has given us impetus to study these worms at the molecular level. Experiments were therefore carried out with this main goal in mind.

Experimental Procedure

A. Absorption measurements

Fifty planaria, obtained commercially, were washed with distilled water and dropped one by one into liquid nitrogen. The frozen worms were ground in a mortar maintained below freezing temperatures by setting it on top of a slab of dry ice. The powder, mostly ice, was allowed to thaw, and the resulting suspension was transferred to a 30-ml pear-shaped flask. Lyophilization of this suspension yielded a brown-grey powder, about 40 mg. The powder was resuspended in 1 ml of 0.05 M potassium phosphate, pH 6.8, with the aid of a hand homogenizer. The heavier material was allowed to separate by settling for a few minutes and the supernatant suspension was decanted. The latter was centrifuged to collect the particulate material, which was then resuspended in 4 ml of potassium phosphate buffer. The absorption spectrum was measured in a cuvette with a 0.3-cm path length, with the scatter-transmission attachment for the Cary Model 14.

B. Cell disaggregation methods

For each of the procedures listed below, five planaria were washed twice with 0.05 M, potassium phosphate buffer, pH 6.8, and then homogenized in a Virtis homogenizer at 0°C, either in 1 ml of phosphate buffer for 90 sec and at 10,000 rpm (homogenate A) or in 1 ml of a mixture of 0.5 M sucrose, 0.1 M potassium phosphate, and 0.01 M EDTA, all at pH 7.4, for 30 sec and at 10,000 rpm (homogenate B). All samples were examined subsequently under light microscope from 240× to 1,200× magnification.

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1. J. B. Best, Sci. Am. 208, 54 (1963).

2. J. V. McConnell, A. L. Jacobson, and D. P. Kimble, J. Comp. Physiol. Psychol. 52, 1 (1959).

3. W. C. Corning and E. R. John , Science 134, 1363 (1961).

Procedure 1. a. One live worm was added to 0.2 ml of homogenate A.
b. One live worm was added to homogenate A and one drop of toluene was layered on top of the mixture.
c. One live worm was added to homogenate A to which had previously been added 25 μg of dihydrostreptomyocin sulfate.
d. One live worm was added to homogenate A to which had previously been added 200 units of penicillin.
e. One live worm was added to a heat-treated homogenate A (steambath for 5 min, then cooled to room temperature).
All these mixtures were allowed to stand for more than 30 hr at room temperature.

Procedure 2. Bacteria from experiment 1a above were streaked on an agar plate. At least two types of bacteria were observed, one rod-shaped and the other oval-shaped. Each was inoculated into separate nutrient broths; only the rod-shaped type grew. From 500 ml of nutrient broth and after 2 weeks of growth, 1.5 ml packed volume of bacteria was obtained. (All the foregoing experiments were conducted by Mrs. Theresa Andrews.) These bacteria were washed once with 0.05 M, pH 6.8 potassium phosphate buffer. Finally the bacteria were suspended in 5 ml of distilled water and sonically ruptured for 5 min at 9 kc and 0°C. The sonicate was centrifuged at 2,000 rpm for 30 min in a clinical centrifuge; one live worm was added to the supernatant and incubated for more than 24 hr. A similar experiment was performed with a smaller amount of bacteria, but using the Virtis homogenizer plus glass beads at 40,000 rpm for 5 min at 0°C to rupture the bacteria.

Procedure 3. A sucrose concentration gradient was constructed in a 5-ml Lusteroid tube by carefully layering 1 ml of sucrose solution buffered at pH 8.0 with 0.01 M tris, starting with sucrose concentration of 2.5 M and decreasing in steps of 0.5 M to 1.0 M. The tubes were transferred gently to the cold room (about 5°C) and allowed to stand for 24 hr. One ml of sample was then layered on top of this gradient and the tube was placed into an SW39L rotor (swinging bucket) and centrifuged for 1 hr at 39,000 rpm in a Spinco Model L ultracentrifuge. Bands of substances formed during centrifugation were carefully withdrawn with the aid of a long thin needle attached to a hypodermic syringe and examined under a light microscope.

Samples for layering on top of the gradient were the following: (a) Worms ground in an isotonic buffered sucrose solution (see homogenate B above) (b) squashed worms treated with 0.25% trypsin for 4 min at room temperature, and (c) remainder of squashed worms after treatment b, digested a further 30 min with 0.25% trypsin. In the last two cases, the cells plus debris were collected by centrifugation in an International refrigerated centrifuge, Model PR-2, at 2,500 rpm for 10 min. The precipitates were resuspended in 1.5 ml of 0.5 M sucrose, 0.1 M potassium phosphate, and 0.01 M EDTA, all buffered at pH 7.4.

C. Ultracentrifugal analysis of planaria homogenate

Fifty worms were ground in a Virtis homogenizer with the aid of glass beads at 10,000 rpm for 5 min at 0°C in 1 ml 0.01 M tris, pH 7.6. The homogenate was centrifuged at 2,800 rpm for 30 min in a refrigerated centrifuge, yielding 2.4 ml of a slightly turbid supernatant. To this supernatant was added NaCl to make a final concentration of 0.1 M. The concentration of protein was estimated to be about 4 mg/ml. Studies of the sedimentation rate were performed in the Model E Spinco ultracentrifuge at 29,500 and 42,040 rpm, using the schlieren optics.

D. Chromatographic examination of a worm homogenate using Sephadex G-200

All operations were carried out near 0° C. One ml of the slightly turbid supernatant used in the analytical ultracentrifugal experiment was added to a column of Sephadex G-200 (1 cm in diam by 16.5 cm long) which was previously equilibrated with pH 7.6, 0.01 M tris. This buffer was also used to elute the material, and 1-ml fractions were collected in consecutively numbered tubes. The absorption of each fraction was measured from 230 to 300 mμ. Certain fractions were combined and dialyzed against 0.01 M pH 7.6 tris at 5° C for about 24 hr, with three changes to fresh buffer during this operation.

Results and Discussion

A. Absorption spectrum

Two distinct peaks, one at 500 mμ and the other at 410 mμ, were observed in the absorption spectrum of the insoluble particulate fraction of homogenized planaria. The absorbance per 0.3-ml light path of each of these peaks was between 0.05 and 0.10 for an estimated 5 mg/ml of insoluble solid (dry weight). Other lesser peaks were not detected, owing to the large amount of scattering. Although planaria have eyespots, we are not certain that these pigments are localized in them, since pigments appear also to reside in other parts of the worm's body. These results indicate that an "action spectrum" of these worms (i.e., response to light of different wavelengths) should be possible. Ultimately we hope that we can find conditions in which these worms can give maximum response to colored light.

B. Cell disaggregation

The basic idea underlying this approach is to determine the localization of information in various types of cells. If there is a unique localization, then the next step will be to isolate the information carrier from these cells and transfer it, one would hope, to untrained animals.

Very few intact free-floating cells were obtained when planaria were ground with a Virtis homogenizer, either in buffer or in buffered isotonic sucrose. Most prominent were the clumps of cells attached to rubbery membranous material. A living worm added to such a homogenate in 0.05 M phosphate died within a few hours and was subsequently disintegrated after more than 30 hr at room temperature. The presence of large amounts of bacteria was observed. (Worms did not survive in 0.05 M phosphate alone, but in this case, after 30 hr standing, no disintegration was apparent, nor were there many bacteria.) The disintegration was virtually complete--i.e.,

no rubbery membrane-like material remained. In addition to rod-shaped and oval-shaped bacteria, free-floating cells plus clumps of cells were evident. That the worm was not digested by the enzymes of the homogenized worms is shown by experiments with homogenates layered with toluene and by experiments with heated worm extract. The intact worm added to the toluene-layered homogenate did not disintegrate into cells or clumps of cells after standing for more than 30 hr, though initially the intact worm structure was destroyed by the toluene. On the other hand, the untreated homogenate (control), the antibiotic-treated homogenate, and the heat-treated homogenate showed complete disintegration of the added intact worm, along with the usual signs of bacteria, free-floating cells, and cell clumps.

Cell-free extracts in tris buffer of these bacteria grown in nutrient broth failed to attack the worm. In fact, the worm remained alive after several hours, and even after death the worm did not disintegrate up to 24 hr later.

The question always arises whether there are small amounts of free-floating cells among the rather massive amounts of cell debris obtained from various methods of disaggregating the cells. One way of separating and concentrating these cells is to use density-gradient centrifugation. When this method was applied to worms disaggregated by (a) homogenization in buffered isotonic sucrose, (b) short-time trypsin treatment, and (c) prolonged trypsin treatment, three main bands of particulate materials were observed. The first, second, and third bands were located near the 1.0 M, 1.25 M, and 2.0 M sucrose regions, respectively. The second band appeared to have most of the cells, although in all cases the yields were very low and the cells were grossly contaminated with the rubbery tissues of the worms.

Owing to the low yields of cells obtained by straight homogenization and by tryptic digestion of the planaria, the use of bacteria is probably the best. How the bacteria will affect the information remains to be determined. A projected experiment would be to disintegrate the worms with bacteria for 30 hr, centrifuge to collect the cells plus clumps of cells, wash this fraction several times with buffer to free it from bacteria, and finally separate the cells from the clumps by density-gradient centrifugation.

C. Ultracentrifugal examination of a worm homogenate

As expected, the worm homogenate is heterogeneous; however, surprisingly little, if any, high-molecular-weight materials were found. Since schlieren optics were used for these observations, this method was not very sensitive. The bulk of the material appears to have sedimentation coefficients of about 3 or 4, corresponding to molecular weights between 10,000 and 100,000, depending on other parameters.

D. Sephadex G-200 column chromatography of the soluble planaria extract

This column should separate substances of molecular weights greater than 200,000 from those with molecular weights less than 200,000. Figure 10-1 shows results of an experiment with the water-soluble homogenate of worms after passing through Sephadex G-200 and eluting with tris buffer. Two peaks are evident, the first one coming out with the holdup volume of the column (about 7 ml) and the second one eluting 9 to 10 ml later.

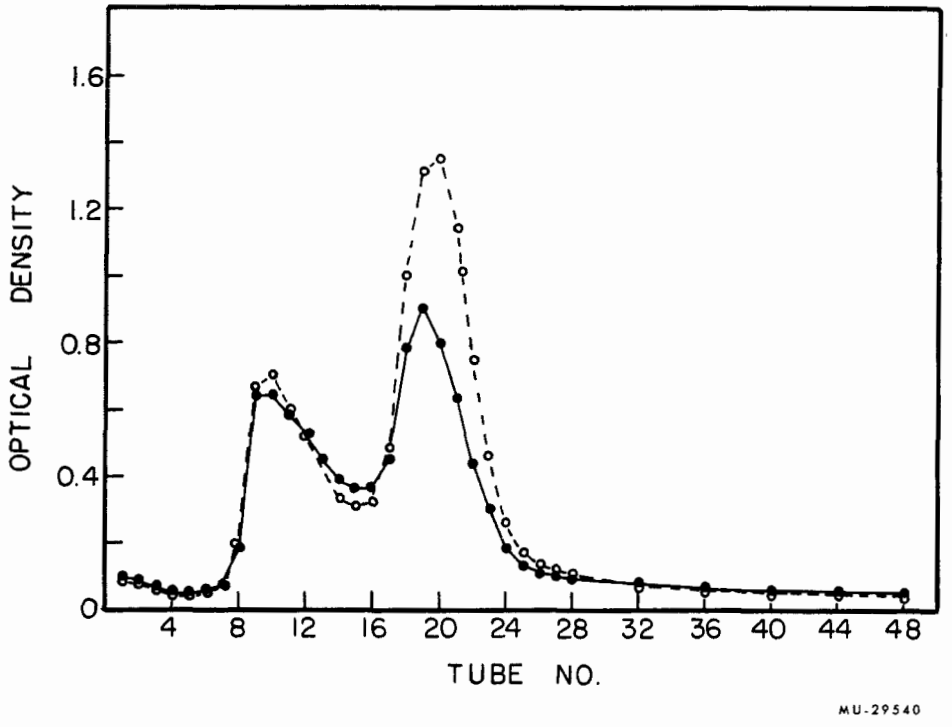

Fig. 10-1. Chromatographic pattern homogenate of Planaria, clarified by centrifugation at 2,800 rpm for 30 min in International refrigerator centrifuge, Model PR-2, passed through G-200 Sephadex column.

●: Absorbance at 280 mμ

○: Absorbance at 260 mμ.

The first peak probably contains materials of 200,000 molecular weight or more, and the second peak contains those of less than 200,000 molecular weight. The peak tube number 10 is relatively rich in 260-mμ absorbing material and perhaps indicates high nucleic acid content. A convenient measure of nucleic acid content is given by ratio of absorbance at 260 mμ (D_{260}) to that at 280 mμ (D_{280}). Hence, tube number 10 has a D_{260}/D_{280} of 1.1 and tube number 15, 0.84.

The second peak has even larger D_{260}/D_{280}, but a large contribution to this ratio is due to nucleotides and nucleosides. Thus, if tubes numbers 18 to 22 are combined, the absorption spectrum of the pooled sample shows a peak at 250 mμ with a D_{260}/D_{280} of about 1.4. After dialysis for 24 hr, the absorption maximum shifts to 255 mμ and the D_{260}/D_{280} drops to 1.3. The absorbance of equivalent amounts of solution also changes from 0.85 to 0.45 at 280 mμ, indicating that almost half of the material in the second peak is of low molecular weight.

One other fact is noteworthy. Although there is apparently a large first peak, the schlieren system failed to detect this material during the ultracentrifugation. The results could be explained in terms of the first peak's being mostly nucleic acids. The use of an ultraviolet absorption optics system for studying the sedimentation of purified material from this peak would seem the most likely to bear fruit.